THE THECAL GLAND IN THE GUINEA PIG OVARY[1]

W. T. STAFFORD, R. F. COLLINS AND H. W. MOSSMAN
Department of Anatomy, University of Wisconsin, Madison

TWO PLATES (TWELVE FIGURES)

INTRODUCTION

Since the isolation and chemical identification of the estrogens by Allen, Doisy et al. in 1924, the exact source of the estrogenic hormone has been sought. Ascheim ('26) and Westman ('34) in a series of experiments concluded that the theca interna was the site of formation of the estrogenic hormone. Mossman ('37) demonstrated a cyclic development of the theca interna of the pocket gopher (Geomys bursarius) which reached its greatest size at or near ovulation. From the cytology of this thecal gland he postulated that it was the source of estrin. Corner ('38) upon reviewing the evidence came to the conclusion that there is a strong indication that the usual site of production of the estrogenic hormone is the theca interna of the graafian follicle. He suggested that, "Hormone production probably begins when the follicle is small and continues as it grows large." A thecal gland has been observed in ripe follicles of several orders and families of mammals, but the study of the full ovarian cycle in all these animals has not been completed. A list of the animals in which this has been observed follows:

Order: Rodentia		
Family:	Sciuridae	Squirrels of many species
	Muridae	Mice and rats
	Erethizontidae	Porcupine
	Aplodontidae	Boomer
	Leporidae	Rabbits

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Order: Carnivora		
	Family: Canidae	Dog
	Felidae	Cat
	Mustelidae	Skunk
Order: Insectivora		
	Family: Talpidae	New world and old world moles
	Soricidae	New world and old world shrews
Order: Chiroptera		
	Family: Molossidae	Mexican bat
Order: Artiodactyla		
	Family: Bovidae	Cow and sheep
	Suidae	Pig
Order: Primata		
	Family: Cercopithecidae	Rhesus monkey
	Tarsiidae	Tarsius (Hubrecht collection)

In no animal was there observed so great a development of the thecal gland as in the pocket gopher, but in all, there was a change from embryonic, spindle-shaped cells about the follicles in early proestrus, to polyhedral cells with granular cytoplasm near and at estrus. Hill and Gatenby ('26) have even described this phenomenon of maximum glandular development of the theca interna near ovulation in the Monotreme, Platypus (Duck-bill), and have postulated a functional significance for the theca at this time. The theca interna is better developed in the human ovary than in most mammals and the enlargement of its cells in ripe follicles has been described (Strassmann, '23 and Shaw, '25), but no glandular significance was attached to this tissue. These changes in the theca interna have also been recorded in the mouse and rabbit by Deanesley ('30), and in the ferret by Robinson ('18) although he called them interstitial cells. Warbritton ('34) described lutein cells in the theca interna of the ewe at estrus which degenerated at the time of ovulation. Still more recently Dawson and Friedgood ('40) showed in the ovary of the cat that the

cells of the theca interna undergo a local hypertrophy at estrus. These cells then degenerated in the 24-30-hour interval between copulation and ovulation, probably because the need for estrin is less.

The following observations were made on the guinea pig, Cavia cobaya, because of its easy availability as a common laboratory animal and in the hope that this investigation might stimulate other workers to follow the cytology of the theca interna of other species through the estrus cycle with the aim of studying enough mammals to determine whether this glandular differentiation at estrus is of universal occurrence.

MATERIAL AND METHODS

A colony of sixty-five adult female guinea pigs was observed, and from this number, nineteen were selected for study of the thecal gland. The animals were protected as much as possible from any factors which might interfere with the normal reproductive cycle. The diet was balanced and adequate in vitamin C.

The animals were followed through several estrus cycles to determine the regularity of the cycle and to determine as closely as possible the onset of estrus. From these data calculations were made to determine the time at which the animals were to be sacrificed.

To determine the onset of estrus several methods were tried. The method originally proposed by Stockard and Papanicolaou ('17) and since elaborated by Young ('37), involving the use of the vaginal smear technique was attempted but for our purpose allowed too much latitude in timing the onset of estrus. The copulatory response method described by Young ('37) was employed and found to be more satisfactory in determining the actual onset of estrus. This reaction as explained in the paper cited, consists in the assumption of the typical copulatory position, i.e. straightening of the back and presenting of the pudenda, in response to stroking the back and vulva with the fingers, or by actual mounting by the male

after the vaginal membrane has ruptured. It occurs almost invariably at the beginning of estrus and is said to be due to the synergistic action of estrone and progesterone produced in the ripe unruptured follicle. Spontaneous mounting activity was found to be too variable to be an adequate criterion of the onset of estrus.

Each animal in the entire colony was examined twice daily; at 7:00 A.M. and at 4:00 P.M.; for evidence of ruptured vaginal membrane. If the vagina was found to be "open" the animal was segregated and immediately examined for the copulatory response as outlined above.

It was desired to obtain animals in the proestrus state, at estrus, and at definite periods following the onset of estrus. Animals desired in the proestrus state were examined through several (at least six) cycles until definite information as to the onset of estrus could be obtained. Careful records were kept. If the cycles were found to be regular it was felt that it was reasonable to predict the onset of the next estrus. Such predictions were made, the animals sacrificed at 5-day, 4-day, 3-day, 2-day and 1-day intervals proestrus, i.e. before the next anticipated estrus.

For animals obtained at estrus, similar predictions were made, the animals watched closely and tested repeatedly for the criteria of estrus. As soon as the copulatory response was positive, its validity as a criterion of estrus was checked by acceptance of the male, the presence of sperm in the vaginal smear and the presence of a copulatory plug (Harmon, '31). Postestrus stages were obtained by determining the onset of estrus as described above, segregating the animals and sacrificing them at desired intervals.

As would be expected, there was considerable variation in the anatomical stage of ovaries from animals estimated to be in the same stage by this method. In the last 3 proestrus days this error seemed to be as much as 2 days when the morphology of the ovaries was compared. For instance, two supposedly 2-day proestrus specimens had ovaries essentially like the 3-day female, and one presumed 24-hour proestrus animal had

corpora lutea of about 24 hours. The post copulatory stages also varied from the expected stage, but never more than could be accounted for by an error of 24 hours. These errors are not due to the inaccuracy of the copulatory response as a test of estrus, but merely indicate individual variations in the length of the successive cycles, and missed responses due to failure to check the animals often enough.

The ovaries described and illustrated are selected cases in which the estimated stage from the data on the cycles and that from study of their morphology seemed to correspond most closely. So far as the history of the thecal gland is concerned an exact chronological estimate of the stages is relatively unimportant; their relative age very important. The latter we based on anatomical evidence from the follicles and corpora lutea. We feel sure this has been done accurately.

The animals were killed by illuminating gas and autopsied immediately. The ovaries were removed and fixed in either Zenker's fluid with 2% commercial formalin or Levi's solution which contains osmic acid [2]. Usually one ovary was fixed in Zenker's fluid and the other in Levi's solution. The ovaries were serially sectioned at 10 micra. Those sections fixed in Zenker's fluid were stained with Harris' hematoxylin and eosin. Those fixed in Levi's solution were triple stained with Biebrich's Scarlet, Orange II and Fast Green, thus avoiding any dark stain which could be confused with osmicated granules. The sections fixed in Levi's solution were covered with neutral balsam to assure against fading the osmic acid stained lipoids.

The entire series of sections of each ovary was examined for follicular development, corpora lutea, and the status of the theca interna.

[2] Levi's Solution Formula

Zenker's stock solution		94 parts
Potassium dichromate	25 gm.	
Mercuric chloride	50 gm.	
Distilled water	1 L	
Commercial formalin (neutralized over CaO and filtered)		1 part
Osmic acid 2% in distilled water		5 parts

DESCRIPTION AND DISCUSSION OF THE THECAL GLAND AND ITS DEVELOPMENT

A. In immature non-vesicular and vesicular follicles, (figs. 1–4)

Non-vesicular follicles are those showing at least one layer of polyhedral cells surrounding the intact ovum and include all those follicles in progressive development to the formation of the follicular antrum.

Immature vesicular follicles are those from the beginning of the follicular antrum to the beginning fragmentation of the discus proligerus and cumulus oophorus which in our series occurred sometime between 48–24 hours before the next anticipated estrus.

About these follicles the theca interna consists of circumferentially arranged cells, closely packed and embryonic in appearance with elongated nuclei and scanty cytoplasm. There are no osmic staining granules in the cytoplasm, although the osmic stain is seen in old corpora lutea elsewhere in the same sections, thus proving no fault in the staining technique. Many mitotic figures are present. The number of thecal cells increases as maturity is approached, until the period between 48 and 24 hours before the predicted estrus. At this time the individual cells of the theca interna begin to increase in size and multiplication practically ceases. The width of the theca interna remains approximately the same. This phenomenon results in an actual decrease in the number of cells comprising any one radius of the theca interna and is probably due to compression and stretching by the enlarging follicle. This period then, between 48 and 24 hours proestrus is the time of transformation of the embryonic theca interna into the specialized thecal gland.

B. In mature vesicular follicles (24 hours proestrus and at estrus, figs. 5–8)

Mature follicles are characterized by a large follicular cavity, a definitely radiate corona radiata and a cumulus

oophorus and discus proligerus which show at least beginning separation and fragmentation. These follicles are usually peripherally located and may show an incipient rupture point. It is at this period that we have used the term "thecal gland" instead of theca interna to designate what we consider a functional glandular structure. It shows the following characteristics: (1) The cells have lost their circumferential arrangement to become irregularly distributed, and have assumed polyhedral instead of spindle shapes. (2) Very few mitoses are seen. (3) Their nuclei have become larger, more spherical and vesicular. (4) The cytoplasm is increased in amount and has a granular appearance although secretory granules cannot be identified with certainty. (5) Osmic acid stained droplets make their first appearance between 48 and 24 hours proestrus and occur in the cytoplasm as tiny, discrete globules. These droplets remain without increase in number or size throughout estrus. They may represent secretion granules. (6) Congestion becomes marked with many leucocytes within the vessels and in the intercellular spaces of the thecal gland.

C. In recently ruptured follicles (1 hour postcopulation, fig. 9)

The most striking difference between mature unruptured and recently ruptured follicles is the marked increase in caliber of the blood vessels and in thickness of the thecal gland. Both phenomena are probably due to the release of the intrafollicular pressure. Luteinization of the granulosa seems to begin almost immediately, but the differentiation between the thecal and granulosa cells is still clear.

D. In the corpora lutea (1 day to 3 days postestrus, figs. 10–12)

Typical lutein cells make their appearance by 24 hours postestrus and are well developed at 48 hours postestrus. The thecal gland becomes progressively more difficult to recognize after 24 hours postestrus so that by 72 hours postestrus it cannot be identified as a separate structure. The internal

limiting membrane which existed between the follicle and the theca interna is disrupted and there is vascularization of the corpus luteum by vessels from the thecal gland. Little pycnosis of thecal gland nuclei can be observed in the guinea pig, although both nuclei and cytoplasm become markedly smaller after rupture of the follicle. We cannot explain the complete disappearance of the thecal gland, but it is possible that at least some of the cells are incorporated into the corpus luteum. Loeb ('06) said that the theca interna cells of the guinea pig ovary enlarged soon after follicular rupture and remained distinct from the granulosa only until about 20 hours post-copulation, after which they mingled with the granulosa lutein cells to form the corpus luteum. Corner ('19) described a similar thecal history in the domestic pig. Warbritton ('34) maintained that in the ovary of the ewe the theca interna cells enter only temporarily into the formation of the corpus luteum and that the lutein cells present in the theca interna at the time of ovulation degenerate. From observations on Geomys and other mammals we favor this view, but must admit that the guinea pig ovary does not yield conclusive evidence on this point.

The area formerly occupied by the thecal gland becomes replaced by the fibrous connective tissue capsule of the corpus luteum. It is probably derived from the theca externa and the stroma of the theca interna. Dawson ('41) states that the capsule of the corpus luteum of the cat may be composed of residual theca interna together with the less well defined theca externa.

E. In atretic follicles

Atresia occurs in follicles of all ages from the immature primary follicles to nearly mature follicles. The first changes discernible in those marked for atresia are absence of mitotic figures, first noticeable in the follicular and then in the thecal cells. Shortly after this, pycnotic nuclei become prominent in the follicular cells and irregularly shaped droplets staining with osmic acid appear in their cytoplasm contrasting with

the round discrete droplets seen in the larger active follicles. The ovum may show pycnosis and large irregular droplets which stain with osmic acid, or in older follicles it may give off polar bodies, and then proceed to fragment or even undergo early cleavage.

There is no evidence of the formation of a thecal gland about atretic follicles in the guinea pig. The thecal cells remain embryonic, even in those follicles which nearly reach maturity before undergoing atresia. As atresia sets in the theca interna cells cease multiplying and quickly transform into interstitial cells. As the follicular cavities become lost and the follicles shrink in size, their sites are marked mainly by these masses of interstitial tissue. It would not be amiss to call these, "interstitial glands." The details of the development of the interstitial tissue of the guinea pig ovary will be described in another paper.

CONCLUSIONS

The thecal gland of the guinea pig ovary can be recognized from the mature vesicular follicle up through rupture and during the early corpus luteum formation. During the growth of the follicle, until between 48 and 24 hours proestrus the theca interna is composed of embryonic appearing cells which are actively multiplying as evidenced by the many mitotic figures present. From the period between 48 to 24 hours proestrus through rupture of the follicle, and to at least 1 hour postestrus the cells of the thecal gland stop active multiplication, increase greatly in individual size, show a granular cytoplasm and simulate a glandular organ. Congestion of the thecal gland is increased greatly just before estrus and reaches its peak immediately after rupture. Shortly after estrus, the thecal gland begins to disappear and cannot be recognized at 72 hours postestrus. The fate of the thecal gland cells is not clear.

Since mature thecal gland tissue is present in the guinea pig ovary only near and during the period of estrus, it is

reasonable to assume that this tissue is the most probable source of the estrogenic hormone.

The theca interna of the atretic follicles never assumes the typical thecal gland structure, but instead transforms into interstitial tissue.

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PLATE 1

EXPLANATION OF FIGURES

All sections are magnified 770 × and were cut at 10 micra. All sections are oriented with granulosa or lutein tissue above. The approximate internal and external extent of the theca interna and thecal gland has been indicated by marks at the right margin of each figure. Elements which might be mistaken for osmicated droplets are indicated as follows: b, blood vessel; m, mitosis or recently mitotic cells.

1 Theca interna of an immature vesicular follicle in an ovary 5 days proestrus fixed with osmic acid. The cells are embryonic, spindle shaped and arranged circumferentially about the membrana granulosa. Mitotic figures are numerous. Nuclei tend to be elongate. Osmic acid stained droplets are scarce, although lutein and interstitial cells throughout the section are well impregnated. Several osmic impregnated interstitial cells show at the lower portion of the figure. (Female no. 44, rt. ov., sl. 3, sect. 8).

2 Theca interna of an immature vesicular follicle in an ovary 4 days proestrus showing morphology as described in figure 1. Zenker. (Female no. 53, lt. ov., sl. 11, sect. 14).

3 Theca interna of an immature vesicular follicle in an ovary 4 days proestrus fixed with osmic acid. Shows very few osmic acid stained droplets in the cytoplasm of the thecal cells, although they are fairly numerous in the granulosa and lutein cells. (Female no. 53, rt. ov., sl. 4).

4 Theca interna of a growing vesicular follicle in an ovary 48 hours proestrus. The thecal cells show some loss of circumferential arrangement and the nuclei are more spherical. Mitoses of the theca interna cells are common. There is considerable increase in vascularity. Zenker. (Female no. 48, lt. ov., sl. 3, sect. 25).

5 Theca interna of a nearly mature follicle in an ovary 24 hours proestrus. Shows theca interna transformed into the thecal gland. The cells have lost much of their circumferential arrangement about the membrana granulosa and are polyhedral in shape with marked increase in amount of cytoplasm. The cytoplasm is granular in appearance. Nuclei are larger, more spherical and vesicular. No mitotic figures are seen. The vessels of the thecal gland are less well filled than at 48 hours proestrus, but intra- and extra-vascular polymorphonuclear leucocytes are common. This is indicative of the resolution of a mild inflammatory process. Some of the nuclei interpreted as polymorphonuclear leucocytes may be pycnotic thecal gland cell nuclei. Zenker. (Female no. 42, lt. ov., sl. 11, sect. 15).

6 Theca interna of a nearly mature follicle in an ovary 24 hours proestrus (same animal as fig. 5) fixed with osmic acid, showing the first appearance of numerous, large osmic acid stained droplets in the cytoplasm of thecal cells. Note that there is a similar change in the granulosa cells. Other changes as described in figure 5. (Female no. 42, rt. ov., sl. 13, sect. 14).

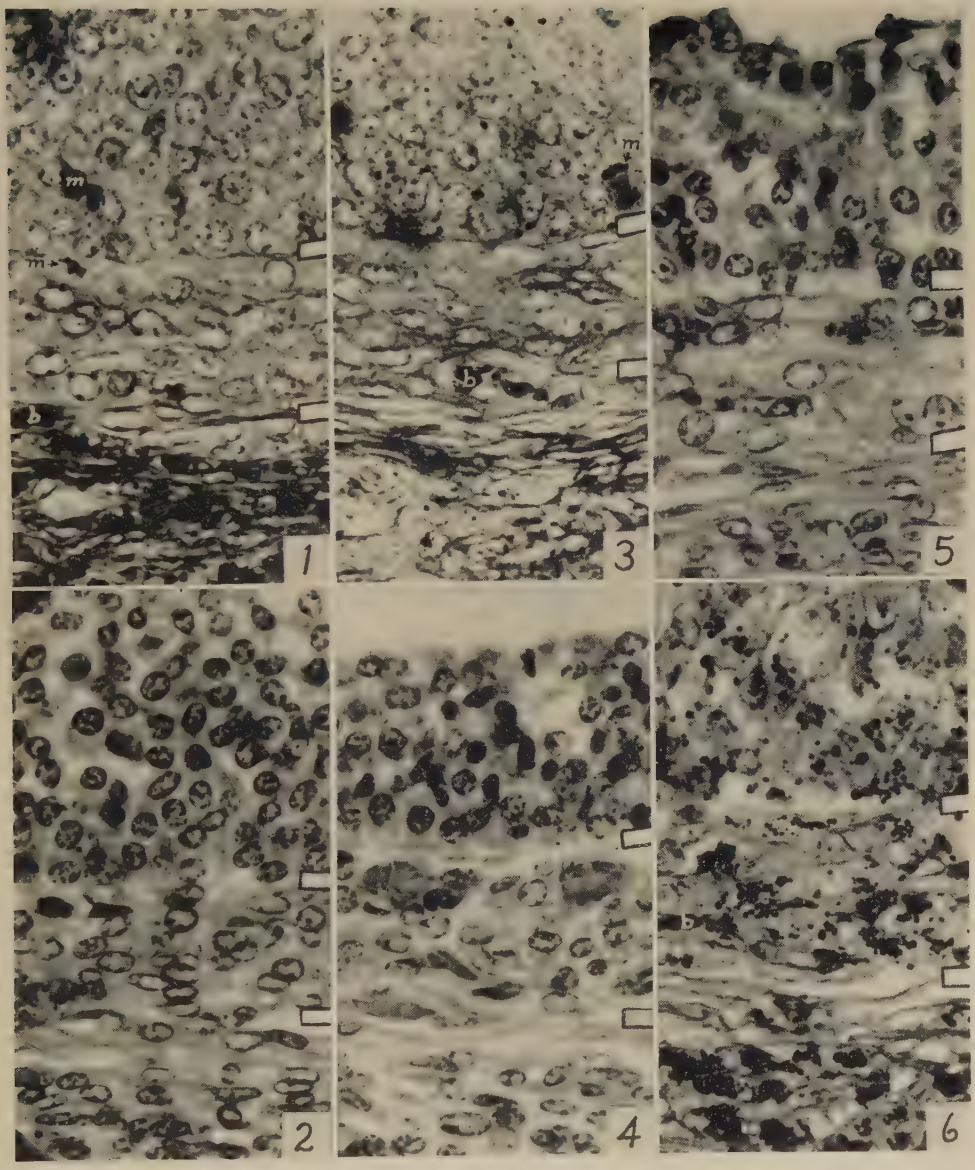
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PLATE 2

EXPLANATION OF FIGURES

All sections are magnified 770 × and were cut at 10 micra. All sections are oriented with granulosa or lutein tissue above. The approximate internal and external extent of the thecal gland has been indicated by marks at the right margin of each figure, except for figures 11 and 12, in which the thecal gland or theca interna is no longer present. In these last two figures a single mark shows the junction of the lutein tissue with its capsule of connective tissue. Elements which might be mistaken for osmicated droplets are indicated as follows: b, blood vessel; m, mitosis or recently mitotic cells.

7 Thecal gland of a mature follicle in an ovary obtained at estrus. The cells are definitely polyhedral with granular cytoplasm. The changes seen in figure 6 are more advanced. The vessels are more dilated than in the ovary at 24 hours proestrus and leucocytes are even more numerous. There is some possible pycnosis of thecal gland cell nuclei, although confusion with leucocytes is hard to avoid. The entire structure is definitely glandular in appearance. Even the cells of the theca externa are somewhat enlarged, but they do not have the glandular characteristics of the interna (Female no. 101, lt. ov., sl. 4, sect. 7).

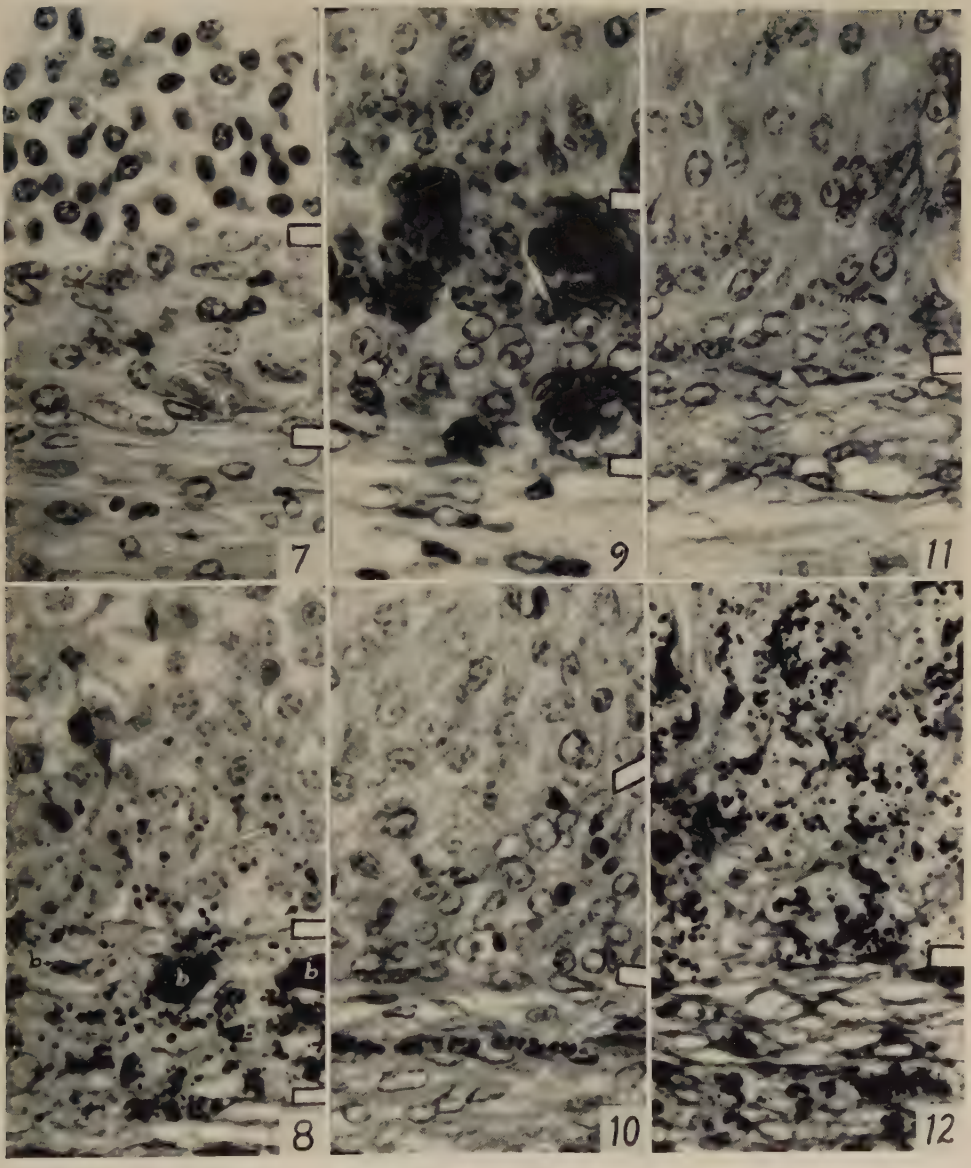
8 Thecal gland of a mature follicle in an ovary obtained at estrus (same animal as fig. 7). Fixed with osmic acid. Shows large osmic acid stained droplets in the cytoplasm of the theca cells. (Female no. 101, rt. ov., sl. 18, sect. 12).

9 Thecal gland of a recently ruptured follicle in an ovary 1 hour postestrus. The thecal gland is seen to be markedly thickened and the vascular channels increased in caliber, due to release of intrafollicular pressure after follicular rupture. There are very few leucocytes and no signs of pycnosis of the gland cell nuclei which, however, are much more crowded than just before rupture indicating a definite shrinkage of the cytoplasm of these cells. There are no mitoses in the thecal gland. Zenker. (Female no. 58, lt. ov., sl. 3-B, sect. 16).

10 Thecal gland of a young corpus luteum in an ovary 24 hours postestrus. The lutein cells are well developed and often seen in mitosis. The blood vessels are extending into the corpus luteum from the thecal gland. The thecal gland is of irregular thickness and its boundaries are indistinct. Its cells are much reduced in size and although their nuclei are crowded there is no evidence of pycnosis. They no longer have a glandular appearance. Mitoses are rare in the region. The vessels are not markedly dilated, but polymorphonuclear leucocytes are again plentiful, several showing in the figure. Zenker. (Female no. 49, rt. ov., sl. 5, sect. 6).

11 Border of a well developed corpus luteum in an ovary 48 hours postestrus. The thecal gland now is almost unrecognizable as such. It is replaced by fibrous connective tissue forming the capsule of the mature corpus luteum. Mitoses are common in the lutein cells; one is shown here. The fate of the thecal gland cells is not clear. Zenker. (Female no. 30, rt. ov., sl. 12, sect. 15).

12 Border of a mature corpus luteum in an ovary 72 hours postestrus fixed in osmic. The thecal gland structures are entirely replaced by the fibrous connective tissue capsule of the corpus luteum. Osmic acid stained droplets are numerous in the lutein cells. (Female no. 73, rt. ov., sl. 3).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ANDROGEN PRODUCTION DURING PREGNANCY AND LACTATION IN THE RAT[1]

M. W. BURRILL AND R. R. GREENE

Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago, Illinois

ONE TEXT FIGURE AND TWO PLATES (FIVE FIGURES)

Increased excretion of estrogen during pregnancy has been demonstrated repeatedly in mammals. D'Amour, Funk and Glendenning ('36) found estrogen in the urine of the pregnant rat and Beerstecher ('41) showed that the concentration increases during pregnancy reaching a maximum on the last 4 days (eighteenth to twenty-first days). Differentiation of reproductive structures in the rat occurs mainly in the latter part of the embryonic period and is proceeding rapidly at the time the estrogen excretion is maximal. Although experimental work has shown that estrogens administered to the pregnant rat cause a marked feminization of the male embryos (Greene, Burrill and Ivy, '40), it does not necessarily follow that the quantity of maternal estrogen normally present is sufficient to modify the sexual development of the embryo. However, we have previously postulated that, during pregnancy in the rat, there is a production of androgen sufficient in quantity to counteract any possible effect of the estrogen on the embryos. Two assumptions were involved in this postulate (a) that androgen is produced during pregnancy in the rat and (b) that estrogens and androgens are mutually antagonistic in their effects on the embryos. The latter assumption has already been shown to be valid inasmuch as experiments have demonstrated that androgens, administered to the pregnant

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rat in appropriate amounts, prevent the usual feminizing effects of administered estrogens on the male embryo and, similarly, estrogens prevent the masculinizing effects of androgens on the female embryo (Greene, Burrill and Ivy, '41, '42). The present investigation was undertaken to test the first assumption, i.e., to determine whether or not the pregnant rat produces androgen.

In a preliminary report (Burrill and Greene, '41 a) it was stated that a physiologically detectable quantity of androgenic substance is present in the female during certain periods of pregnancy and lactation. This statement was based on the finding of androgenically stimulated ventral prostates in female rats during the latter part of pregnancy and the first part of the lactation period. This study has since been extended and the complete results are presented here.

METHOD OF STUDY

The animals used in this study were white rats of a special strain in which a high percentage of females have ventral prostates [2]. One-hundred and sixteen ventral prostates from these females have been examined microscopically for evidence of androgenic stimulation. The series includes prostates representing each day of pregnancy and each day of lactation through the twentieth day. The method of dating the pregnancies was as follows. Routine search for copulation plugs was made each morning. The day on which a plug was found was considered day 0 of pregnancy and the day following, which was the first day on which any prostates were removed, was considered day 1 of pregnancy. With this method of dating, 78% of these females delivered on day 22 of pregnancy. Ventral prostates removed on this day were taken within 20 hours after delivery and were included in the postpartum series. Thus the day after delivery was considered day 1 of lactation.

[2] The progenitors of this strain were kindly provided by Dr. Emil Witschi. The descendants of these animals have been inbred in our laboratory for more than a year and a half.

Usually a whole lobe of the prostate was removed. When both right and left lobes were present these were taken at separate laparotomies. After fixation in Bouin's fluid, the prostates were serially sectioned at 10 μ. Serial section was considered advisable because the histological character of the prostate is not always uniform. Considerable variation between different regions of the same lobe is sometimes found (see fig. 2).

The acinar epithelium of the ventral prostate of the rat is known to respond in a very definite manner to the presence or absence of androgenic substances. Estrogens, although they may affect the prostate in other ways, do not produce or maintain a response of the epithelium such as is evoked by androgens. The epithelium of the ventral prostate therefore offers an ideal criterion for the detection of androgenic substance, particularly in a state such as pregnancy when high concentrations of estrogen are present.

In a fully functional prostate, one under the influence of androgenic stimulation, the acinar epithelium is high columnar and the nuclei are basally located. Just distal to the nucleus, there is an area in the cell which takes up less strain than the rest of the cytoplasm (fig. 6). These so-called "light areas" are considered evidence of secretory function and thus diagnostic of androgenic stimulation (cf. male prostate: Moore, Price and Gallagher, '30). They disappear in the male prostate soon after castration and appear in the completely atrophic gland within 48 hours after administration of an androgen (personal observation of the authors). In the present study, prostates were considered functional only if light areas were found on microscopic examination.

Not all of the non-functional prostates, however, could be considered completely atrophic. An atrophic or non-stimulated prostate has a very characteristic appearance. The acini are uniformly small and the epithelium is low, generally the cells are little or no wider than the nuclei. The epithelium is also somewhat irregular in appearance and there is apparently no discrete basement membrane separating the epithelium from

the surrounding connective tissue cells (fig. 3). In some of the non-functional prostates the acini were not uniformly small and the epithelium was not atrophic, but fairly high although without light areas. In some regions the epithelium was undergoing marked proliferation as indicated by the number of mitotic figures present and its pseudo-stratified appearance (fig. 4). In the transition from the atrophic to the functional state the prostate undergoes not only an increase in the height of the acinar epithelium, but also a marked increase in the size of the acini, therefore it is reasonable to assume that a period of proliferation is passed through in this transition. Prostates showing marked proliferative activity, but no light areas can be considered intermediate in character between the atrophic and fully functional states, but nevertheless representative of an early response to androgenic stimulation. With continued stimulation these prostates would undoubtedly show full function. In some prostates the presence of light areas in some acini and active proliferation in others is indicative of such a progression in response.

Other non-functional prostates showed no proliferative activity. They were, however, not completely atrophic since the epithelium in most or all of the acini was fairly high and regular. These were considered to be regressing from a previous functional state, the light areas having already disappeared in response to a withdrawal of androgenic stimulation or a reduction below the threshold level.

FINDINGS

After microscopic examination of each prostate it was classified as (1) functional, (2) proliferative, (3) regressing or atrophic. These different conditions are shown in the photomicrographs. Figure 1 shows the result of classification of the prostates removed on each day of pregnancy and lactation. Because of the individual variability in prostates removed on the same day of pregnancy and because of the different numbers of prostates removed on different days, the data have also been arranged according to percentages of each type of

prostate for each 3-day period of pregnancy. These percentages are diagrammatically shown in the figure.

Functional prostates were found on days 2, 3 and 5 of pregnancy. A proliferative prostate was also found on day 1. In the 3-day period including days 7, 8 and 9 all the prostates were regressive or atrophic. During the remainder of pregnancy the percentage of stimulated prostates steadily increased for each 3-day period until, in the last period including days 19, 20 and 21, all prostates were stimulated (i.e., either functional or proliferative).

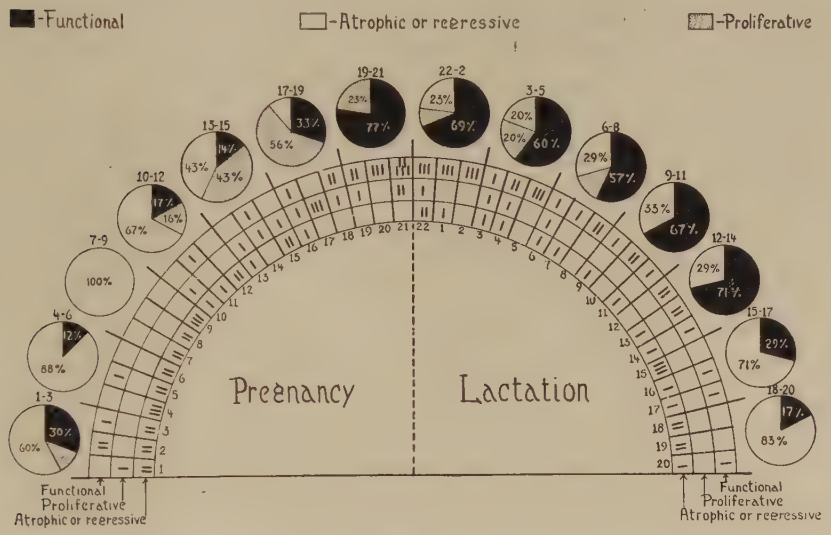

Figure 1

As previously stated, the prostates which are classified as proliferative probably represent an early stage in the change from an atrophic condition to a functional state. In the diagram it is apparent that from the 10- to 12-day period to the end of pregnancy there is a gradual shift in percentages, the per cent of atrophic or regressed prostates gradually diminishing and the per cent of proliferative and functional prostates gradually increasing. There is also a shift in percentages from the proliferative to the functional condition.

During lactation functional prostates were found on each of the first 15 days (days 0 through 14 of lactation). The percentage of stimulated prostates, both functional and proliferative, was also high for the 3-day periods covered by these 15 days. In the last two periods, days 15 through 20, the percentages of stimulated prostates were markedly lower and the percentages of atrophic or regressed prostates correspondingly greater.

INTERPRETATION OF FINDINGS

If the prostate can be considered an end organ which varies in histological character in accordance with the presence or absence of androgenic substance in the body, certain interpretations of the data are warranted. The so-called functional state of the prostate (presence of light areas) indicates the presence of a quantity of androgen sufficient to produce a trophic effect on the prostate. Atrophic prostates may not indicate an absolute lack of androgen, but certainly suggest that the quantity of androgen within the organism is below the threshold level of the gland. In some females there is apparently an effective amount of androgen present during the early period of pregnancy. Toward the end of pregnancy there is apparently an effective amount present in all females. On days 6, 7, 8 and 9 of pregnancy all prostates were negative with respect to androgenic stimulation. This finding, considered in the light of the steadily increasing per cent of stimulated prostates in subsequent periods, leads to the interpretation that there is a period when androgen is not produced, or more conservatively, when the amount of androgen in the organism is below the threshold level of the prostate. The onset of this period and its duration undoubtedly varies in individuals. In one case an effective level was indicated as late as day 16 and in another case a non-effective level was indicated as early as day 11. This period of relative or absolute androgen lack must, however, be only temporary since in all cases the prostates reflected the presence of an effective level at the end of pregnancy.

Another factor which should be taken into account in any interpretation of the data is that the condition of the prostate at the time of its removal does not reflect the hormonal status of the organism at that particular time, but represents a previous status. A certain time interval is required before a change in the hormone concentration in the organism can register an effect on an end organ such as the prostate where marked histological changes are involved. Tislowitz ('39 a) found that the latent period in the response to testosterone was 24–25 hours determined by the onset of mitotic activity in the seminal vesicle of colchicine treated castrated mice. We have found (unpublished observation) that 48 hours were required for restoration of the atrophic rat prostate to the functional state (light areas) after an injection of testosterone propionate. This checks well with Tislowitz's finding that testosterone propionate, a slower acting substance than testosterone, has a slightly longer latent period, 27–31 hours for the onset of mitotic activity in the mouse seminal vesicle. On the basis of these determinations it may be assumed that a period of 24–48 hours would probably elapse between the time when the androgen reached an effective level in the body and the occurrence of recognizable histological changes in the prostate.

The functional state in a prostate from a female only 2 days pregnant (i.e., approximately 48–60 hours after copulation) therefore indicates that there must be an effective concentration of androgen following closely on the ovulation which marks the beginning of a pregnancy. Since, in the male, light areas in the prostate may persist for 3 days after withdrawal of androgen, i.e., castration (unpublished observation of authors), lack of light areas in all prostates on the sixth and seventh days indicates that the early effective level of androgen in some females must be of very short duration and must quickly drop below the threshold level of the prostate.

In view of the fact that all prostates taken from females during the last 5 days of pregnancy gave evidence of being androgenically stimulated, it was surprising to find that on

the day of delivery and on subsequent days of lactation some of the prostates were completely negative. It is well known that, in the rat, an ovulation occurs shortly after delivery. Long and Evans ('22) have stated that this ovulation usually occurs between the sixteenth and twenty-fourth hours after delivery. The changes leading up to this ovulation may cause alterations in the hormonal status of the female. It is not our intention to state that these changes necessarily affect the androgen level of the pregnant female at term. However, if such a change did occur, assuming that it would be in the direction of an apparent decrease in androgen level or even a temporary cessation of production, it is reasonable to suppose that it might be reflected in the condition of the prostate during the first few days post partum. Since all biopsies on day 22 were taken within 20 hours after delivery, the occurrence of two regressed prostates on this day indicates that the hypothetical androgen decrease causing their regression undoubtedly occurred before delivery. Although, as has been stated, light areas may still remain in the male prostate even 3 days after complete removal of androgen, it cannot necessarily be concluded that the regressed prostates found on the day of delivery represent a withdrawal of androgen 3 days earlier, i.e., on day 19 of pregnancy. It is possible that the regressed prostates found on day 22 may have been among those which had not yet reached complete function late in pregnancy and thus, in response to a drop in the androgen level even as late as day 21, might show regressive changes on the following day.

This hypothetical drop in androgen production very late in pregnancy, if present in all animals, is probably only temporary and may not have registered an effect on those prostates which had already attained the functional state, since a high percentage of prostates on the first days of lactation gave evidence of full function (light areas).

It is, of course, possible that no drop in the androgen level occurs toward the end of pregnancy, that the finding of negative prostates during the early post-partum period is an ex-

pression of individual variability. This view is supported by the fact that, although the percentage of stimulated prostates was high for the first 2 weeks of lactation, there were negative prostates scattered throughout this period.

Two of the prostates removed on day 21 of pregnancy were from females not of the special prostate strain, but of our regular laboratory stock in which the incidence of female prostates is 14%. The fact that both of these prostates were functional demonstrates that the female prostate, when present, is capable of responding to androgenic stimulation and that the findings reported are not peculiar to the special strain used as material for this study.

DISCUSSION

The findings demonstrate that a physiologically effective amount of androgen is produced during certain periods of pregnancy and lactation in the rat. Since estrogens have been claimed to have certain stimulatory effects on the ventral prostate, the question may arise as to whether the findings indicate a stimulation by estrogen rather than by androgen. In a survey of the literature, not a single instance has been found in which estrogens produced light areas in the epithelium of the ventral prostate. This response is specific for androgenic substances, therefore it is reasonable to assume that the presence of light areas in the female ventral prostate during certain periods of pregnancy and lactation constitutes proof for an effective concentration of an androgenic substance at these times.

In the presentation of findings, prostates showing evidence of proliferative activity in the epithelium were considered to represent an early stage in the response of the gland to androgenic stimulation. It is extremely unlikely that this proliferation is a response to estrogen rather than androgen. There are reports in the literature of a stimulatory action of estrogens on the prostatic epithelium, but these particular reports concern the prostate of the mouse which responds to continued estrogen administration by an epithelial squamos metaplasia.

In the rat, metaplasia has not been produced in the acinar epithelium of the ventral prostate. Korenchevsky and Dennison ('35) produced metaplasia in the coagulating glands and dorsal prostate of castrated rats with estrone, but found no evidence of metaplasia in the ventral prostate. Weller, Overholser and Nelson ('36) produced metaplasia in the prostates of normal and castrate mice with daily injections of an estrogen, but found no metaplasia in prostates of rats treated similarly even for longer periods. In our own laboratory (unpublished data) treatment of castrated rats with 100 gamma of α estradiol daily for 21 days caused no metaplasia of the ventral prostate.

Although Tislowitz ('39 b) found some mitotic activity in the prostatic epithelium of colchicine treated castrated mice after treatment with estrogen for 1 week, no similar experiments on the rat prostate have been reported. Since metaplasia readily occurs in the mouse prostate in response to estrogen, it is reasonable that the first indication of such a response would be increased mitotic activity. The proliferative female prostates found in the present study, however, showed no evidence of a metaplastic type of response. Although the epithelial cells were actively multiplying, the epithelium was definitely not truly stratified, but retained its columnar type, the crowding of cells producing a pseudostratified appearance. One of the most intensely active glands is shown in figure 4.

During the progress of the present study Takewaki ('41) published experimental results on the mouse which correlate with our own findings in the rat. Seminal vesicle transplants in female mice, after colchicine treatment, showed increased mitotic activity in the epithelium during the second half of pregnancy. Although secretory granules were never found in the seminal vesicle epithelium, Takewaki interprets the increase in mitotic activity as indicative of androgenic rather than estrogenic stimulation on the basis of control studies with estrogen treated animals. Since he did not give details of dosage in these control studies, it is impossible to compare

his results with those of Tislowitz ('39 b) who obtained some increase in the mitotic activity in the seminal vesicle epithelium of male mice treated with various estrogens. Unfortunately, Takewaki's observations did not include the stage of pregnancy preceding the sixth day. It would be interesting to know whether or not the mouse, like the rat, would show evidence of androgenic stimulation in this early period.

Also in 1941 (a), Crabtree published observations on the renal cortex of mice during pregnancy and the puerperium. In the kidney of the mouse there are normally two types of Bowman's capsules. In one, the parietal epithelium is made up of flattened cells, and, in the other, the epithelium is cuboidal. Crabtree previously reported ('41 b) that the percentage of cuboidal capsules is greater in the male mouse than in the female of the same age. Crabtree's studies ('41 c) and those of Selye ('41) indicate a correlation between the amount of androgen present and the relative number of capsules with cuboidal epithelium. Crabtree found a marked increase in the percentage of cuboidal type capsules during the first half of pregnancy and a gradual decrease to control level in the last trimester. If these findings may be considered indicative of androgen level during pregnancy in the mouse, the lack of demonstrated androgenic activity during the latter part is at variance with Takewaki's finding in the same animals and does not correspond with our observations in the rat. The data offered by Crabtree, however, were obtained from ten animals, each observed on a different day of pregnancy. Marked individual variations such as we have observed in the rat prostate are recognized as a probability by this author, but are not considered as the possible cause for the curve obtained. Actually the conclusion that there is a decrease in androgenic effect on the renal corpuscles in the last third of pregnancy is based on the findings in one animal killed on the eighteenth day. However, even if the renal corpuscles failed to show evidence of androgenic effect in late pregnancy, it is not necessarily true that no androgen is present during this period. The possibility remains that androgen may be pres-

ent, but is antagonized in its effect on the capsular epithelium by a high concentration of estrogen. Crabtree does not mention whether or not such an antagonism between the two hormones in their effects on the capsular epithelium has ever been demonstrated.

In the monkey, according to a recent study by Dorfman and Van Wagenen ('41), excretion of both androgen and estrogen is increased in pregnancy over the non-pregnant level. Only the latter part of pregnancy was studied, but the experimental procedures employed strongly indicate that the androgen found during this period of pregnancy in the monkey is largely produced by the adrenal.

Concerning the identity of the androgenic substance in the pregnant and lactating rat, we have no actual information. Since progesterone has been shown to have an androgenic effect in the rat (Lamar, '37, '40; Greene, Burrill and Ivy, '39; Greene, Burrill and Thomson, '40), it is necessary to consider the possibility that androgenic manifestations during pregnancy and lactation may be due to this substance. It is not, of course, necessary to assume that the androgenically active substance produced during early pregnancy is identical with that produced late in pregnancy. It may be progesterone at one time and another androgen, perhaps even from a different source, at the other time.

The source of the androgen produced during pregnancy and lactation in the rat has not been determined by the procedure employed in this study. The ovary, the adrenal cortex and the placenta are possible sites of production. However, unless it is assumed that the androgen of lactation has a different origin from that of pregnancy, the placenta must be excluded as a probable source. Evidence from many sources indicates that production of an androgenic substance is a function not wholly foreign to either the ovary or the adrenal cortex. In the immature male rat and mouse the adrenal has a capacity for producing androgens (Howard, '39; Burrill and Greene, '39; '40) and in the immature female rat this capacity is apparently shared by the ovary and the adrenal (Burrill and Greene,

'41 b). In the human, androgen has been definitely shown to originate in certain masculinizing tumors of the adrenal cortex (Crooke and Callow, '39). Recovery of androgens from the urine of human castrates of either sex tends to inculpate the adrenal as the source (Callow, Callow and Emmens, '38). Various substances having androgenic activity have been isolated from bovine adrenals (Adrenosterone-Reichstein, '36; progesterone-Beal and Reichstein, '38; 17-β-hydroxyprogesterone-Pfiffner and North, '40).

The quantity of androgen produced by the pregnant and lactating female rat has not been ascertained by our method of study. Although the estrogens in the urine of the rat have been quantitatively determined, such determinations do not necessarily indicate the actual quantity of circulating estrogen. The fact that androgen is produced concomitantly with estrogen late in pregnancy lends credence to the theory of hormonal balance and it is pleasant to believe that the relative quantities produced are such that one nullifies the effects of the other on the embryo. As a consequence of such a balanced condition the embryo would normally undergo sexual development virtually unimpressed by the maternal hormones. It should be emphasized that this theory of maternal hormone balance was devised to fit the circumstances in the rat and the evidence which has been offered for this theory may apply only to the rat.

SUMMARY AND CONCLUSIONS

The ventral prostates from female white rats of a special strain have been removed during pregnancy and lactation, and have been examined microscopically for evidence of androgenic stimulation. The resulting data provide evidence that during certain periods of pregnancy and lactation androgenic substance is present in amounts capable of stimulating the prostatic epithelium. During the early part of pregnancy (days 1 to 5) some females apparently have a physiologically effective quantity of androgen. In the next period (days 6 to 10) there is no evidence of androgen as judged by the histo-

logical character of the prostate. This has been interpreted as a cessation of production or a decrease in production below the threshold of the prostate. During the remainder of pregnancy, conditions in different individuals varied, but the general trend indicated an increase in androgen production. During the last 4 days (17–21) all prostates were stimulated. On the first 15 days of lactation there was apparently an effective quantity of androgen in most individuals, but on the last 6 days of lactation most prostates reflected the absence or subthreshold level of androgen. The identity and source of the androgen have not been determined in the present study.

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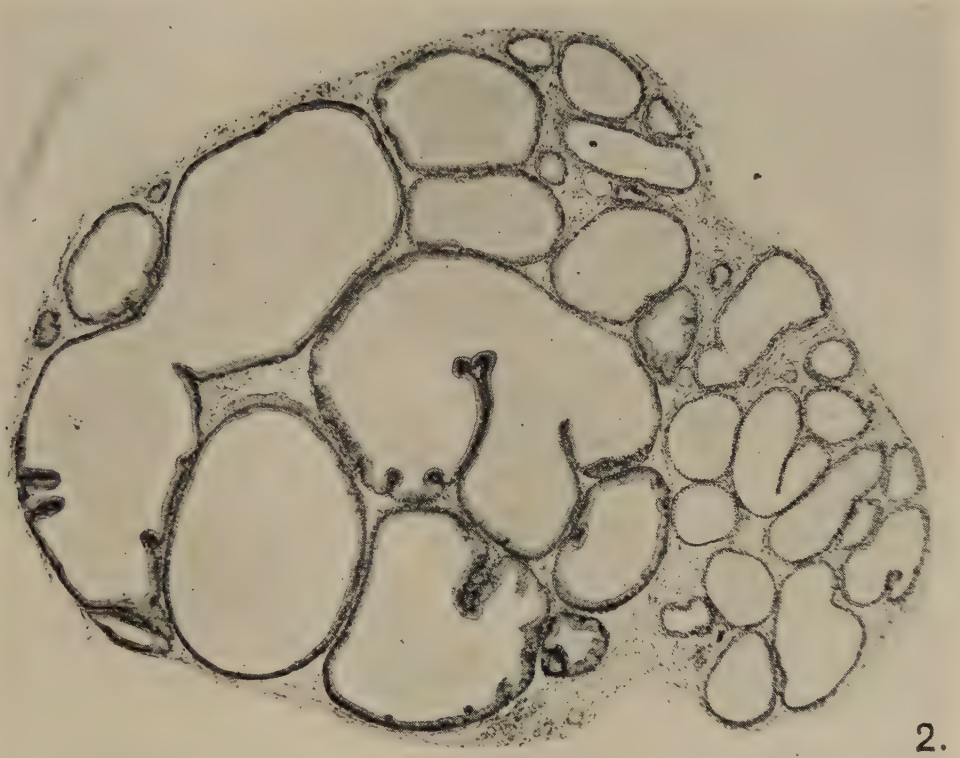
PLATE 1

EXPLANATION OF FIGURES

2 Ventral prostate taken from female on day 5 of lactation. 55 ×. This section shows the variation which may exist in a single gland; the acini on the left of the photomicrograph have high, functional epithelium (see fig. 6), while those on the right are atrophic.

3 Ventral prostate taken from female on day 8 of pregnancy. 55 ×. Note the relatively large amount of interacinar stroma, the small size of the acini and the low epithelium, all characteristics of an atrophic gland.

4 Ventral prostate taken from female on day 21 of pregnancy. 55 ×. This prostate is representative of the type called proliferative. The epithelium is high, but pseudostratified and mitoses are frequent. Figure 5 shows the epithelium of such a gland in greater detail.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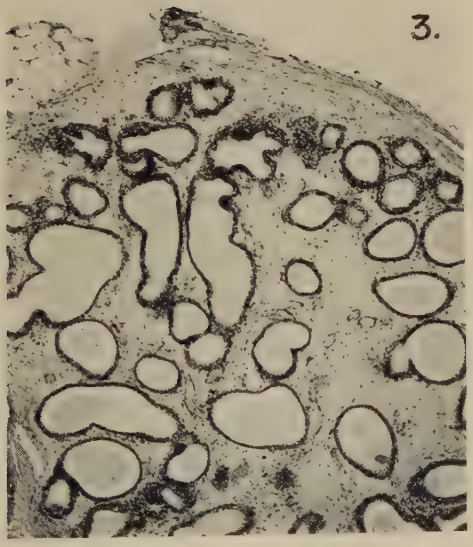
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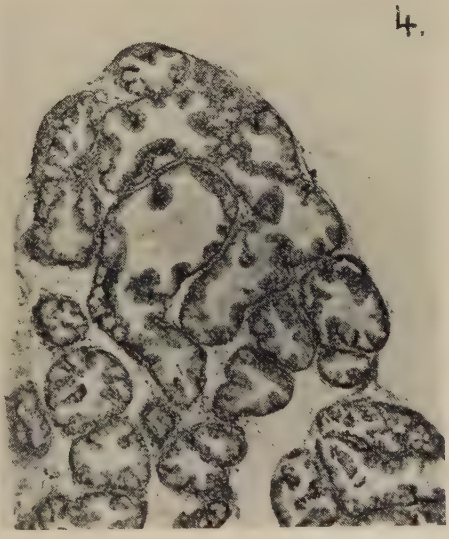
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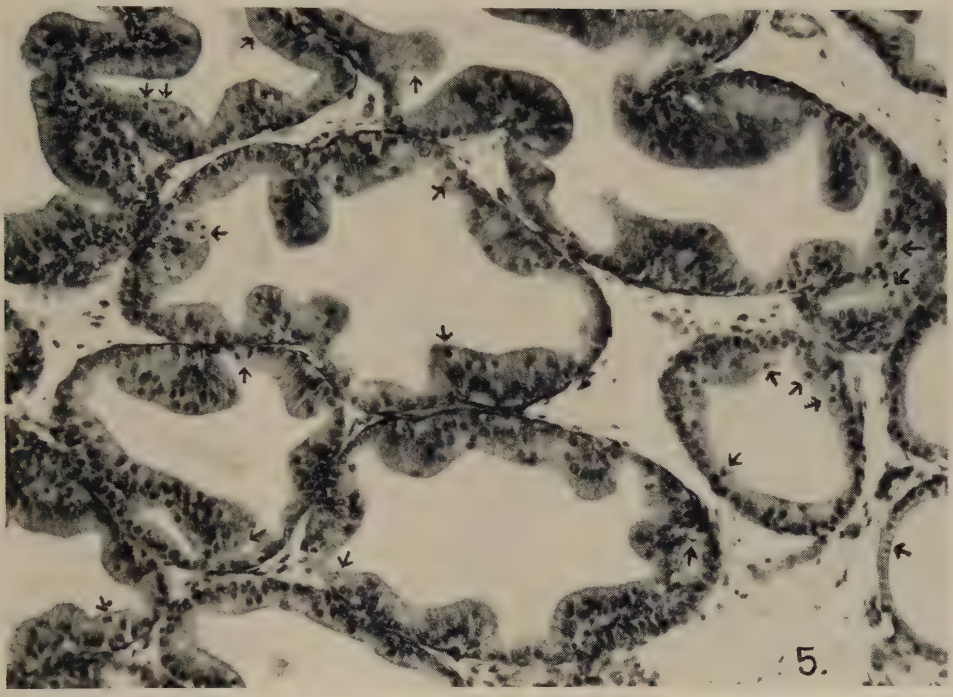
PLATE 2

EXPLANATION OF FIGURES

5 Ventral prostate taken from female on day 20 of pregnancy. 190 ×. Although this animal had received no colchicine, the mitoses are fairly frequent (designated by arrows). Light areas may be seen where the epithelium is simple columnar; where activity is intense the epithelium is pseudostratified and light areas cannot be seen.

6 Detail of figure 2. 385 ×. High columnar epithelium with light areas and basal nuclei characteristic of the fully functional type of gland.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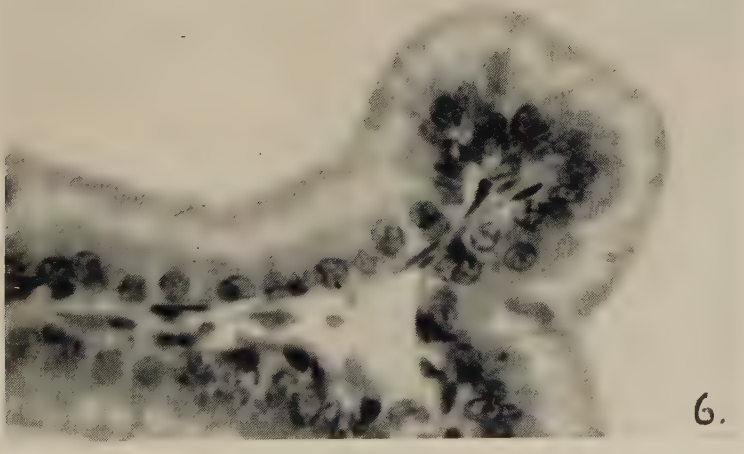
6.

THE INTRODUCTION OF BIOLOGICAL STAINS: EMPLOYMENT OF SAFFRON BY VIEUSSENS AND LEEUWENHOEK

FREDERIC T. LEWIS
Department of Anatomy, Harvard Medical School, Boston, Massachusetts

While reading the highly repetitious letters of Leeuwenhoek on the fibers of flesh, to find what kernels of truth may be buried in the considerable chaff, the writer came upon the following passage, apparently overlooked in the history of staining. It is in a letter sent to the Royal Society from Delft, August 21, 1714, included in the Opera Omnia, but apparently never published by the *Praenobiles Domini* to whom it was addressed. Leeuwenhoek had long since learned to make sections, being, in Dobell's opinion, "one of the first — if not the very first" to employ them. "Some which he cut with his own hand 'by means of a sharp shaving razor' are still in existence. They were enclosed in a little packet affixed to an early letter (1 June, 1674) and according to his own description are (1) Cork; (2) White of a writing pen [parings from a quill]; (3) Bits of the optic nerve of a cow, cut crosswise; (4) Pith of elder."[1]

Leeuwenhoek was examining the muscle fibers of a decidedly fat cow for comparison with those of an 8-year old lean one, which he thought might yield information of practical importance, when he wrote:—

Cum verò dictæ fibrillæ, utpote in tenuissimas lamellas sectæ, admodum essent pellucidæ; adeoque ægrè dignosci possent; tantillum croci maceravi in vino adusto. Hoc vino

[1] Dobell ('32, p. 333). But Robert Hooke, of course, with his "sharpen'd Penknife" had cut sections of cork 10 years earlier.

particulas carneas, ut delineàtori spectabiliores essent, tantillùm perfudi; quæ mox rutilabant colore luteo.[2]

This result is easily obtained. Let a gram of Spanish saffron (the dried stigmas of Crocus, twisted off, with enough of the style to hold them together) simmer half an hour in tap water; filter, and apply the stain to a section of muscle fixed in formaldehyde. The fibers indeed glow with a golden yellow color. Though nuclei remain almost imperceptible, the histological pattern is decidedly clearer. Masson recommended saffron in 1911, unaware of Leeuwenhoek's priority, when he wrote:—

Le Safran officinal ne paraît pas avoir été employé jusqu'ici en histologie. C'est cependant un réactif précieux qui jouit d'une affinité très remarquable pour le collagène qu'il teint en jaune d'or brillant. Certains échantillons donnent une coloration spécifique, absolument pure, de cette substance. La plupart teignent aussi, mais beaucoup plus faiblement, les protoplasmas cellulaires. D'où la nécessité de l'employer en combinaison avec un colorant nucléaire bleu et l'éosine ('11, p. 573; and preparations with Masson's triple stain are indeed "fort belles, très démonstratives [3]."")

Leeuwenhoek's use of saffron was in a measure anticipated by Vieussens. In his Neurographia, 1685 (p. 40–41, and presumably also in the 1684 edition not at hand) Vieussens describes tying the carotids in the middle of the neck, and injecting repeatedly into one of them "spirits of wine colored with saffron" (spiritus vini croceo colore infectus). Then on opening the head, only the gray substance is found imbued

[2] Epistolae Physiologicae, being Tom. III of the Opera, Delphis, 1719, p. 102. "Since the said fibrillae, cut into the thinnest possible layers, were so transparent that they could hardly be recognized, I have macerated a little saffron (crocus) in burnt wine. To make the fleshy particles more visible to the artist, I have merely moistened them with this wine, whereupon they were bright with a yellow color."

[3] A misleading note in the Journal of the Royal Microscopical Society (1889, p. 467), headed "Staining Muscle with Saffron," ascribes that procedure to Leven. The Journal's note was taken from "The Microscope," which derived the error, in turn, from the "Medical Chronicle." It was Pfitzner's solution of the modern azin dye *safranin* that Leven recommended in 1888 — far removed from saffron both chemically and in staining properties.

with the saffron color; the natural white of the medullary substance is unchanged. "If the contents of the arteries flowed directly into the veins," he argues, "the gray substance would not be *tinged or stained* by the injected liquid, with which it is always found to have been colored" (cinerea cerebri substantia injecto liquore, quo semper infecta reperitur, nec tincta nec imbuta foret).

For Vieussens, that proved the non-existence of capillaries in the cortex. There, as in many other parts of the body, he said, arteries connect with arteries, veins with veins; but the arteries do not unite with the veins.

Suspecting that such a perparation might be merely a rich capillary injection of the vascular cortex, with no staining, a cat was injected by Dr. E. W. Dempsey, exactly as described by Vieussens. The pia mater became bright yellow; the cortex on vertical section appeared uniformly a duller yellow; and the white substance remained white. The vessels which, when full of blood, contrast with the cortical parenchyma seem to disappear when injected with saffron, since the diffusing or escaping dye imparts the same color to the adjacent parenchyma. Thus Vieussens was misled, by his successful staining, to believe that the arteries discharge their contents into the cortex.

In his Traité du coeur, 1715, Vieussens injected the coronary arteries with saffron. In the pericardium those arteries, he states, go over into veins, but he found also "une infinité de petits filamens ou de conduits lymphatiques-arteriels," some of which pass from arterial walls to venous walls. These connective tissue fibers, stained yellow, he mistook for injected vessels. He found that the saffron penetrated all the tissue of the heart, entering auricle and ventricle, but regarded it wholly as injection, and adds that the demonstration succeeds much better when mercury is used in place of "la teinture de safran." Kellett ('42, p. 47) records that Vieussens, "filling the stomach with a tincture, noticed that the colour diffused through into the gastric veins."

Saffron is an ancient dye, the history of which, with Homeric and Shakespearean citations, is found in Maw's sumptuous "Monograph of the genus Crocus,"[4] but it was rivaled as an early biological stain by the juice of an American berry, called "poke," from corruption of the Indian word "pocan," meaning red. Parkinson in his Theatrum botanicum, 1640, names this weed Solanum "from the likeness of the leaves," and Virginianum since it "groweth in Virginia, New England, &c. from whence the seed and plants were first brought to us." "The Indians therewith doe both colour their skinnes, and the barkes of trees wherewith they make their baskets." The Royal Society hopefully thought that insects feeding upon this redweed might make the color permanent, serving like cochineal insects that feed on prickly-pear. Their colour hath been found, we read, "no whit inferior to that of the Cochineel-Flye" (Philosophical Transactions, 1668, p. 796). Thus when Pierre Magnol (1638–1715), professor of botany at Montpellier and handsomely commemorated in the Linnaean genus Magnolia, undertook to disprove a circulation of the sap, which had been alleged following Harvey's discovery, he made use of pokeweed red. As recorded in the Histoire of the Academy of Paris, 1709, he put the stem of a flowering tuberose over night in the juice of Solanum racemosum mixed with a little water: this juice is the color of lake, and the tuberose became a beautiful shade of pink. The experiment did not involve staining. It was an auto-injection, which produced very simply in plants the effects of the 20th century ink injections of living embryos, without requiring subsequent clearing. Its antecedents were the reputed methods of making lilies red by putting powdered cinnabar in the soil or into the bulb, and of turning roses yellow or any fancied color by watering with appropriate tinctures (see the astrologer-physician Mizauld's

[4] Maw, George. A monograph of the genus Crocus. With an appendix on the etymology of the words Crocus and Saffron, by C. C. Lacaita. London, 1886. M. Kronfeld's booklet, Geschichte des Safrans, Wien, 1892, is in large part derived from Maw.

"nine hundred pleasant devices," gathered from the literature, Paris, 1566).

Magnol's method was so easy that it had a host of followers, and the placing of stems in stain for injection merged into the staining of vegetable tissue with no clear line of separation. Meanwhile Tournefort in his "Institutiones" gave pokeweed its present name of Phytolacca, explaining — "Lacca, as you would call a plant from which a color approaching lake is extracted."

The Jesuit father Nicolas Sarrabat, of Lyons, (1698–1739) immersed the roots of plants in the red juice of phytolacca to find the path of entry of the watery nutriment, and thence to trace its distribution to leaf tips and stamen filaments. He found that the cortex of the roots acquired externally and internally a red color, deeper in the tiny rootlets than in the larger ones. This was confirmed by Bonnet in 1754 and appears to have been a true staining. "After washing the part of the stem which had soaked in the fluid, the color appeared only in the places in the bark where the epidermis was lacking" (Duhamel's review of Sarrabat's paper, 1758, p. 285). Father Sarrabat, after winning the prizes offered by the Académie Royale des Belles-Lettres, Sciences et Arts de Bordeaux for the years 1727, 1728 and 1730, with essays on compass variation, sea salt, and the causes of winds, respectively, was asked not to discourage others by competing further. Accordingly his "Dissertation," which we have been considering, "sur la circulation de la sève dans les plantes" was entered and published under the pseudonym *de la Baisse*. It took the Bordeaux prize in 1733 — "a work to be reckoned with, born of experiment" (Haller) [5].

[5] Unfortunately, so far as known at the Library of Congress, there is no copy of this classic in the United States, and microfilms from Paris or London are not now readily available. Holzner (1884) ventures to assign to Sarrabat a place in the history of staining relying on Haller's abstract, which hardly discriminates between staining and mere injection. For further data as to Sarrabat, see "Bibliothèque de la Compagnie de Jésus," de Backer and Caryon, nouvelle éd. par Carlos Sommervogel, Tome 7, Brussels 1896, p. 650.

Sarrabat's phytolacca was, according to Hoefer (1872, p. 237), the Mexican species — not the common P. americana [6]. As a dye the brilliant juice from the latter, and presumably from both, must be obtained from fresh berries. Dry pokeweed berries yield only a dingy decoction, of acid reaction; but it can stain brown the spiral and annular thickenings of the tracheal tubes which it fills. When fresh, it can heighten the color of red wines, whence the king of Portugal gave orders "to cut and destroy the plants of phytolacca to save the reputation of the wines of his country" (Braconnot, 1807). Doctor Heise of the imperial Gesundheitsamt (1895) tells how to detect it in wines. Others have studied the pigment spectroscopically (Bischoff, in Hilger's report, 1879) and chemically (cf. Muriel Wheldale Onslow's Anthocyanin Pigments in Plants, 1916). Though Lacour (1890) may have succeeded in fixing this fading dye on textile fibers, and though it may equal the juice of certain other berries highly recommended as stains (cf. Lavdowsky, 1884; Claudius, 1899 — Lawson Tait, it may be remembered, preferred red cabbage to carmine) phytolacca has altogether failed of expectations.

Next to be employed as a stain was the ancient madder, Rubia tinctorum, used by Egyptians in coloring mummy cloth, and noted by Mizauld (1566, p. 104) as imparting a red color to the bones of sheep that feed upon it. But he erred in saying that "similarly it can be seen in the boiled and roasted flesh of such an animal, which appears red as eggs boiled in a decoction of the root — their shells become as ruddy as if cooked with bits of Brazil wood." The error as to flesh was corrected by the British surgeon, Dr. John Belchier, in 1736, when he dined on pork served him by a dyer. The bones in that pork were red — the teeth particularly, except their enamel — but "neither the fleshy nor cartilaginous parts suffered the least alteration in colour or in taste." Finding that

[6] Linnaeus in the 1st edition of the Species Plantarum (Holmiae, 1753) described P. americana as having a variety mexicana, with sessile berries. In his 2nd ed., 1762, he made them two species, renamed P. decandra and P. octandra respectively. Though P. decandra is still in common use, we understand that Linnaeus exceeded his authority: *P. americana* is the name to be retained.

the dyer fed his hogs with bran used in cleaning calico of excess color, Belchier gave a cock madder root mixed with fig dust, thus eliminating the metallic mordants of the dyer's refuse. Within 16 days the cock died, and its bones were red (Phil. Trans., 1736) [7].

Duhamel verified Belchier's observations promptly (1739), and reported that he was trying to introduce the tincture of madder into the vessels of plants. But Bonnet (1754) appropriated that suggestion. He found that blanched seedling beans and peas, plunged in the madder infusion, showed appreciable coloring of their roots in less than 24 hours. He preferred to use a green tincture of rose galls, or, best of all, a black ink. Packets of sap-conducting woody fibers were thus brought out, as shown in a crudely figured cross section "grossie au microscope," (to the extent of 11 diam.). "Only weak attempts," Bonnet comments, but the method is "a rich mine." Duhamel (1758), citing Magnol, de la Baisse, and Bonnet, also approved "ces sortes d'injections." He repeated an experiment of Hales (1731, cf. Exp. XII, p. 43–45) who, he says, "ayant coupé une branche à un Poirier chargé de fruit, souda à l'ergot, un tuyau de verre dans lequel il versa de l'Esprit-de-vin camphré. La branche, après en avoir imbibé une pinte, mourut." The odor of camphor, evident in the leaves, was imperceptible in the fruit. In these experimental studies then in vogue, there was very little histological progress.

[7] The story of madder is well told by Prof. Johann Beckmann in his Beyträge zur Geschichte der Erfindungen, Leipzig, 1799, Bd. 4, pp. 41–55. But he regards Dr. Livin Lemmens (1505–1568, who studied at Louvain under Vesalius) as the first to record the effect of madder upon bone. His evidence is a version of Mizauld's statement of 1566 which he found incorporated in a late edition (Cologne, 1581) of the popular work by Lemmens or Lemnius, De miraculis occultis naturae — 1st ed., Antwerp 1559. However, the early editions of Lemnius have not been shown to include that passage — we find madder entered only as a dye for cloth — so that Mizauld seems to have priority in reporting what may have been current information. The extensive later literature of madder is reviewed by G. R. Cameron in "The Staining of Calcium," Journ. of Path., 1930, vol. 33, pt. 2, pp. 929–955.

Georg Christian Reichel (1727–1771) studied sap while a medical student (De succis plantarum, Kiesling et Reichel, Lipsiae, 1752) and thereafter conscientiously read and cited all the botanical literature. Learning of de la Baisse through Bonnet's "Feuilles," Reichel performed ten similar experiments, for which he used scrapings of Pernambuco wood boiled in water for a quarter of an hour, filtered and cooled — an "agreeably red decoction." In this he stood various plants, woody and succulent, some with their roots, and others cut stems, both with and without flowers and fruit. The experiments, which were much alike, led to a single conclusion, viz. that nutritive sap, as shown by the red fluid, was distributed to all parts of the plant by the spiral ducts, wrongly thought by Malpighi, and less definitely by Grew, to convey air (Reichel, De vasis plantarum spiralibus, Leipzig, 1758).

Reichel studied the cross and longitudinal sections of his injected stems with a Lieberkühn microscope — a single lens — of limited power. With steady hand and meticulous care, he drew and engraved the exquisite plate which illustrates his thesis, but which requires a hand lens for satisfactory inspection. It lacks the clarity of Grew's conventional pictures amply enlarged, and has none of the vitality of Malpighi's drawings which Reichel criticizes as crude (rudiori stylo factae). Unfortunately the plate is not in color. But the spiral vessels are described as darkly and excellently stained by the red liquor (egregie tincta); the woody body was lighter red; the parenchymal substance was orange, or sometimes it took a certain yellow (flavedinum quamdam). Bark and pith were generally unaffected. This was differential staining, and Reichel saw it "summo cum gaudio."

Dr. John Hill, reasonably disappointed at non-election to the Royal Society, but malicious in reprisal (see Professor Woodruff's "The Versatile Sir John Hill, M. D."), stained certain plant cells with cochineal as early as 1770, in sections cut on a serviceable microtome. Disclaiming the invention of this "cutting engine" which he figures and describes in

detail [8], and equipped with an Adams microscope made more or less according to his own specifications, he proceeded with a systematic study of the cells, "blebs," and ducts, "vessels," which compose the higher plants (The Construction of Timber, London, 1770). He directly injected some of the largest vessels, and followed smaller ones by partly immersing the upright stem of any plant in a solution of sugar of lead. "When it has stood two days, take it out, and clip off all that part which was in the liquor, and throw it away." The result, in the remaining portion, is what he called a "colourless *impregnation*" of the vessels with lead. By subsequent immersion in a solution of quick lime and orpiment — "the Liquor Probatorius Vini of some of the German chymists: it discovers lead when wines are adulterated with it; and will show it anywhere" — the impregnation becomes "a deep brown," accentuating the dark spots or pits along the vessel walls. Hill's report as to these pits or perforations is perhaps the major outcome of his study. An easier preparation, he found, could be made "with only a tincture of cochineal." "Put half an ounce of cochineal in powder into half a pint of spirit of wine; set it in a warm place, and shake it often, for four days; then filter off the clear tincture. Put an inch depth of this into a cup; and set upright in it pieces of the Rind of Ash, White Willow, and Ozier; prepared, as has been directed, by maceration in water." In the ash, the vessels are "beautifully seen" — they alone are crimson; in the willow, the "colouring liquor" enters also the interstitial spaces; whereas in the Rind of the Ozier, "the whole substance of the Rind is *stained* with it." This seems rightly regarded as the first report of cochineal staining.

In addition to this pioneer staining and impregnation, Doctor Hill notes casually his "method of hardening" (loc. cit., p. 28), which he applied to soft wood fibers obtained separately by his process of maceration. He had a clearing agent also, for dense tissue "being put into spirit of turpentine, will,

[8] It could cut sections 1/1000 of an inch thick, sometimes 1/2000 (= 25 μ–13 μ). See Hill, 1770; also "An Eighteenth Century Microtome," anonymous review, Journ. Roy. Micr. Soc., 1910, p. 779–782.

after a week's standing, become very transparent'' (p. 77). ''Hill was much ahead of his time'' (Conn, '40).

Wilhelm Friedrich von Gleichen, or Gleichen-Russwurm (1717–1783) admired Dr. Hill or he would not have written ''Gleichwie aber Leuwenhöcke, Schwamerdame und Hille selten gebohren werden'': — in the French translation, ''Il paroît rarement des Lewenbocks, des Schwamerdams, des Hill.'' As Hill was presumably the first to use cochineal as a biological stain, Gleichen was the first to employ its rich crimson product, carmine, as reported in his Abhandlung über die Saamen- und Infusionsthierchen, 1778. He thought there would be advantages if he could give an animalcule food that would color its viscera, though aware of the wonderment with which ''enlightened posterity will read — or will no longer read — the poor stuff (den Wust) of our learned opinions.''

''The bones of animals colored by the feeding of madder roots led me to this idea. So I colored some water with carmine, and mixed it with an infusion of wheat in which a swarm of the largest ear-drop organisms [9] and small oval animalcules had been living some months. By the second day I saw my expectations fulfilled, and was not only convinced of the actual swallowing of food, from inner need, by most of these animalcules, but at the same time I learned to know better their interior (ihr Inneres: p. 140).''

In a colored plate (XXIII. b) which he drew himself (mittelmässig,'' nicht schön), the larger organisms contain from few to numerous coarse pink globules, some of which become extruded. Bell animalcules appeared later in this infusion, and one with seven stained vacuoles has also been pictured. Whether the red objects were eggs or excrement Gleichen could not determine, though he watched them patiently for signs of growth or movement [10].

[9] Pandeloquenthierchen (animals shaped like ear-ring pendants) was Gleichen's term: Hill is credited with the name Paramecium.

[10] Turned out of home in early youth, the future Baron de Gleichen had little formal education; but as page at the court of the Prince, as ensign, winning successive promotions in the army and the favor of Friedrich II of Prussia, he acquired a list of courtly titles which burdens the title page of his book here cited. The latter half of his life was an escape to scientific studies, and his contributions to botany, including the discovery of the pollen tube, are important.

Fontana, in his "Observations sur la structure du corps animal" appended to the "Traité sur le vénin," 1781, has left no record of staining any tissue. Carnoy (1884, p. 174) under the heading "Les premiers *essais de microchemie,*" remarks, "Fontana se sert d'alcalis, d'acides; il emploie le sirop de violettes et les couleurs végétables sur le porte-objets." That was in repeating the work of Mead, who had already tested venom with the indicators, syrup of violets and tincture of turnsole; but Fontana went further. He placed the venom and these reagents in drops, presumably on "un porte objets de cristal," and watched them unite under the microscope. But the results were negative: Fontana saw no more in this way than could be seen with the naked eye.

Johann Hedwig (1730–1799) of Hungary, professor first of medicine and then of botany at Leipzig, included in his fundamental work on mosses, in 1782, two irrelevant sections of squash stems, cross and longitudinal respectively. He adopted Reichel's method and exclaims, "See what is found in Cucurbita, Reichelii ad normam, decocto Fernambuccae immissa." In the thinnest possible section, cut with the sharpest knife, placed on a glass slide and viewed with a high-power lens, he pictures ducts "some saturated with the decoction, others less red, some dilutely yellowish." As shown in his Tab. 2, these colors are in the walls of empty tubules; the tissue is therefore stained [11]. Reichel's hair-fine lines have become double contoured walls, mistaken for capillary channels.

Another who followed the same procedure was Johann Christoph Andreas Mayer (1747–1801), best known for his succinct Abhandlung vom Gehirn, Rückmark, und Ursprung der Nerven, 1779. In 1787 he became professor of botany and director of the botanical garden in Berlin. To commemorate this transfer to his "favorite occupation" he presented a "memoir on the vessels of plants" to the Berlin Akademie (1787, but published in 1793). With due credit to de la Baisse, Bonnet, Reichel, and Hill, and using decoctions of Pernambuco

[11] In some copies of this book the plate is not in color.

wood — in one case, Brazil wood — he differentially stained cortex, sapwood, heartwood and pith. Blue, reds and olive contrast and blend in his low-power figures of cross sections, sometimes with slaty, black and drab shadings in the cortex from further staining with aqueous extract of gall-nuts. Injected vessels may be obscured by the spread of stain into the surrounding parenchyma. Several times such effusions spoiled his preparations so that he had to throw them away as unfit to be drawn.

It was inevitable that logwood (Haematoxylon) should soon be used in these injections. Thomas Andrew Knight (1759–1838), in two of his "upwards of a hundred papers,"[12] reported to the Royal Society that he had employed it. On transecting the "runner" of a potato plant leading to a tuber and immersing both cut ends in a decoction of logwood, he found that the color traveled more readily toward and into the tuber than in the reverse direction (1803, p. 288–289).

Later (1808, p. 317) he took various branches and sealed their cut ends with "a composition of calcined oyster shells and cheese . . . covered with a mixture of bees wax and turpentine . . . to effectually exclude all moisture." He continues:

A part of the bark was taken off each branch, in a circle round it, a few lines distant from its lower end, where the tubes had been closed; and each branch was then placed in a decoction of logwood, in a vessel deep enough to cover the decorticated spaces. At the end of twenty hours, or somewhat longer periods, these branches were examined, and the coloured infusion was found to have insinuated itself between the alburnous tubes, in many instances apparently through the cellular substance. This was most obvious in the walnut tree, the young wood of which is very white. The principal object I had in view in making this experiment, was to detect the passages through which I conceived the sap to pass from the bark to the alburnum.

[12] Most famous among them are his descriptions of gravitational experiments with seedlings. Others present many new horticultural varieties of fruits and vegetables which he produced by crossing: he had an estate of "ten thousand acres."

The experiment is easily repeated. Crystals of haematoxylin dissolved in water yield a deep red solution. A poplar stem, sealed with paraffin and superficially girdled, was allowed to stand in it over night. Cross sections through the decorticated band showed blood-orange groups of bast fibers, yellow wood and gray parenchyma. Though primitive, it was assuredly a haematoxylin-stained preparation that Knight produced.

Staining was better understood by Heinrich Cotta (1763–1844), Oberforstrath in Saxony, and devoted to forestry, it is said, from his birth in a hunting lodge. In his sap experiments he used a "red ink." The best of several which he tested was "an extract of Pernambuco wood with some alum but without any vinegar" (1806, p. 4). Thus he introduces into histology a mordant as employed by all dyers [13]. He made the important observation that with a *living* stem no color ascends in the pith or cortex. "Only after the leaves have wilted . . . does the pith become somewhat colored, which is then a biting in (ein Einbeizen) and not a sucking up of the fluid (ein Ansaugen)." Staining is thus sharply distinguished from injection and is regarded as a post mortem occurrence. "With more than a hundred kinds of wood, under most diverse conditions, I made the test and found not a single case where the living cortex would take up the color; only into that already dead can the stain gain entrance." [14] There is no microscopy in Cotta's thesis, unless it be that "durch ein Mikroskop" (p. 64) he saw that "bristle-like" root hairs, which quickly dry in air, regain their original form when wet, even after months of dessication — "eine für die Holzkultur nicht unwichtige Erfahrung."

[13] Unless, with G. M. Smith, ('15, p. 80) we regard Hill's lead impregnation as "a mordanting of the tissues before developing the color."

[14] To what extent there may have been vital staining in these early experiments is doubtful, and Cotta's conclusion is essentially sound. But in 1886 (Bot. Ztg., Bd. 44, col. 120–125) W. Pfeffer announced the deep blue vital staining of unharmed rootlets of several plants — their sap, vacuoles and cytoplasmic granules — with methylene blue, even 1 part in 10 million of water. Several other dyes he found similarly non-toxic and efficient.

At this time Professor Link, of Rostock, investigated the way in which sap (or stain) gets out of the tapering blind upper ends of the vessels to enter the parenchyma of leaf and stem — for he recognized no lateral twigs or pores in the vessel walls (1807, p. 80–81; 1809, p. 21). Choosing plants rich in tannin, he stood the stems in a solution of green sulphate of iron (schwefelsaures Eisen). The reagent ascended in vessels which remained uncolored, but the cells near their ends became blackened, diminishing with their distance from the ducts. The iron diffuses through cell walls — sweats through (durchschwitzt) as Link expressed it. His valuable test for tannin is hardly a stain, however, nor is Raspail's reagent for distinguishing between sugar, oil, albumin and resin. In 1829 Raspail reported that with sulphuric acid the body of the ovary in several grains takes a "handsome purple color," due to its abundant albumin, whereas the surface hairs are yellow, from their sugar content. The ovary of barley thus colored is shown grossly big in Raspail's figure, under low magnification.

In 1830, Christian Gottfried Ehrenberg (1795–1876) prefaced an important contribution on Infusoria with an ungenerous reference to Gleichen's earlier experiments, which he was about to repeat and confirm. Rather a joke, he said of them — "mehr ein Scherz" — like Trembley's attempt to color Hydra. Trembley indeed, in 1744 (p. 126–133), reported success in coloring living polyps. He could make pale forms either red or black by feeding them red spiders or black snails respectively. Colored petals of flowers they would not eat, and attempts to grow them in infusions of blue larkspur and yellow marigolds were fatal. With animal food the color became lodged in "grains" or vesicles in the stomach walls. "Ces vessicules paroissent colorées, parce qu'elles sont pleines, parce qu'elles sont imbibées d'un suc coloré." Trembley figured the "grains" as a swarm of granules, of which there are many layers in well nourished animals: their relation to individual cells was an unsuspected refinement.

Ehrenberg announced that for years he had been attempting to demonstrate the digestive apparatus of infusoria by colored

substances, and had always failed since he used metallic, earthy, or boiled dyes which either killed the animals or were not ingested. He continues:

I used also indigo and lac dye, without thinking that the prepared commercial dyes of that sort were usually mixed with white lead. Recently it occurred to me that that addition might well be the trouble. So I experimented with pure indigo dye and pure carmine, which succeeded perfectly. Under observation the stalked vorticellae devoured this food, and, to my surprise, in a few minutes they filled a number of small round stomachs with it, which until then had not been clear to me. . . . These experiments require organic dyestuffs which do not combine too closely or chemically with water, and that do not affect the substance of the animal, the meteoric water, but only muddy it, through the mechanical admixture of very fine particles. . . . Pure indigo, carmine, and sap green are three very transparent colors, easily recognized under the microscope, which in oft tested service never failed me (1830, p. 22).

Thus Ehrenberg sought particulate matter and not a stain. With his carmine granules he found it "extremely interesting to see the continual whirlpool in the water" made by the cilia of vorticella; and carmine was employed in the investigation of possible cilia by the botanists Professor Göppert and Doctor Cohn, of Breslau, when, in their joint paper of 1849, they reported it a serviceable stain.

Heinrich Robert Göppert (1800–1884), professor of botany, pioneering in plant paleontology — a lover of trees, and public spirited conservationist — found a promising pupil in Ferdinand Julius Cohn (1828–1898) at a time when Jewish students were not allowed to receive degrees at Breslau University. But after obtaining the Berlin Ph.D. in 1847, Doctor Cohn returned to Breslau to win distinction through studies of bacteria and the higher cryptograms. Göppert and Cohn, investigating the protoplasmic rotation (cyclosis) discovered in 1774 by Bonaventura Corti in the green alga Chara, but using the allied Nitella, found within the moving cytoplasm some round bodies beset with projections. These Wimper-

körperchen, they thought, might contribute to the protoplasmic movement, though their processes seemed motionless. "Even when we mixed carmine in the water, we could recognize no trace of an eddy. . . . But the ciliated corpuscles were colored all through, and of all the formations contained in the sap, they alone were strikingly and intensely red, whereby their structure was brought out more clearly. We thought perhaps the water killed the cilia by endosmosis . . . and therefore used a denser sugar- or albumin-containing fluid, but they remained stiff" (1849, col. 688).

These authors used also tincture of iodine. After Colin and Gaultier de Claubry, in 1814, had made known the beautiful blue or violet color acquired by starch grains on combining with iodine, and others had shown that after treatment with sulphuric acid, cell walls of cellulose respond to iodine with that same "herrlich blau," sections of vegetable tissue were plentifully produced which a novice would call stained. Such, for example, fill Plates XV and XVI of von Mohl's "Cuticula der Gewächse" (1842), where yellow or brown cutinized cell walls are distinguished from those of blue cellulose, and the latter contrast with the fine black line of the primary wall. Meyen and Schleiden are regarded by von Mohl (1840) as the originators of that method. Anselme Payen (1795–1871) in one of his numerous contributions to the chemistry of plants (read in 1834, published 1843) described "excessively thin sections of the rootlets of maize, plunged in a solution of protonitrate of mercury," as becoming stained in a few minutes "rose and then red in all their nitrogenous parts." Excluding such chemical studies, whether reasonably or not, from the category of staining, it is customary to stress the differentiation of nuclei from cytoplasm by means of carmine as really establishing the staining process. That was accomplished independently by Corti, Hartig, and Osborne.

The Marquis Alfonso Corti (1822–1876) chose to study the ear, moved perhaps by the beauty of Scarpa's contribution De Auditu; for Scarpa was highly honored at the University of Pavia, where Corti studied under his successors, Panizza

and Rusconi. Then he worked for a few years in the laboratories at Vienna, Würzburg, and Utrecht, becoming favorably known to Hyrtl and Kölliker (cf. Schaffer, '14), before publishing his famous account of the cochlea (1851). Among its 26 pages of appended notes, there are two which describe his use of carmine as a stain. In a detached membranous lamina spiralis, softened by dilute hydrochloric acid, Corti saw dubiously what might be apertures, now known to be foramina for small nerves. He wrote:—

At last I have found a method which has put the existence of actual holes beyond question. . . . I left the lamina for about two hours in a solution of carmine, and then washed it in water. Under the microscope, I found that all its tissue was colored red, being darker where it was thicker. The holes were clearly seen as small oval windows. I could easily be sure that there was really no tissue in the holes, and I could make out their borders with perfect distinctness (1851, p. 151).

Corti's note 10 (p. 144) is more significant:—

To observe clearly the epithelial layer and its elements, it is useful to color it with a solution, half water and half alcohol, in which is put a sufficient quantity of sugar and carmine. . . . The nuclei especially become plainly visible (très visibles) because they take a deeper stain than the rest of the cells.

Corti did not picture the cells so stained, but his figure 6, "Couche épithéliale qui tapisse la cavité du limacon observée dans une solution de sucre médiocrement concentrée," shows their character in black and white. It is customary to slight Corti's clear but brief reference to carmine staining, included in a great paper in an important German journal. Gustav Mann ('02) observes — "It attracted no notice: it was written in French."

Theodor Hartig (1805–1880), Oberforstrath in Brunswick, following the profession of forestry in which his father had been eminent, in 1854 repeated the carmine staining experiments of Göppert and Cohn. He acknowledged that he took up and continued their studies, bringing them, we may add, to full fruition. His results were published in three papers in

the same journal — the well-known Botanische Zeitung. Hartig observed that certain bodies in the cell "like a filter-apparatus hold back the dissolved material, so that they take the saturated color of the dye, even when the dye is diluted to a marked degree" (1854, col. 575). The bodies so stained are especially the nuclei and nucleoli, as Hartig reiterates and italicizes:—

Im Safte des Embryosackes junger Bohnen werden die sehr grossen Zellenkerne und nur diese roth gefärbt (col. 555).

Im Zellgewebe des Stengels, der Wurzel, der Blätter steht die Farbenaufspeicherung in der Regel nur dem Zellkerne und den aus den Kernkörperchen desselben sich entwickelnden Gebilden zu. . . . Die Farbenaufspeicherung ist so bestimmt auf den Zellkern beschränkt, dass. . . . (u. s. w., col. 576).

Unter den verschiedenen in Anwendung gebrachten Farbstoffen behält Karminlösung den Vorzug, einestheils wegen ihrer leuchtenden und sehr hervorstechenden Farbe, deren Aufnahme durch den Zellkern zwar ein sehr tiefes Roth erzeugt, das aber doch immer noch genügend durchscheinend bleibt, um die Form und Grösse der einzelnen gefärbten Körper im Innern des Zellkerns . . . unterscheiden zu können (col. 877).

In the embryo sac of Vicia and Crambe stained with carmine, Hartig saw that the nuclei undergo a very peculiar thread-like or lobate expansion. Especially striking are the filiform and often spirally wound formations — threads staining deep red with carmine (col. 581). Thus staining revealed to him essential features of the then unknown process of mitosis, but he was committed to a "mistaken cell theory" (cf. Sachs, 1875, p. 340), and uses a set of original terms which require a glossary.

Not only did Hartig anticipate Gerlach in the use of carmine by five years, but, as Gierke rightly concedes, "seine Resultate sind eigentlich weitergehend" (1884, p. 71).

Hartig used also litmus, and obtained, in certain bodies, a fine deep stain from the juice of phytolacca! He had a Prussian blue method, and found silver nitrate "ein ganz gutes Färbungsmittel, indem es unter Lichtwirkung dem Zellkern eine

fast schwarze Farbe ertheilt'' (col. 879) [15]. By mixing an iodine solution with the carmine, he made a double stain which colored starch blue, nuclei red, protoplasm (''ptychode'') brown, while the chlorophyll remained green, and the secondary layers of the cell wall, white (col. 878). But there were no figures with these papers. Dr. Hartig mentions that ''drawings had been in the hands of the copper-engraver several months.''

Excellent figures of carmine-stained plant cells, with others not so good, were published by Lord Osborne in 1857. In connection with his paper on ''Vegetable Cell Structure,'' read to the Microscopical Society of London, in June, 1856, he had a plate in color, showing with low power a considerable stretch of wheat root-tip. The deepest carmine is at the apex, where Bonnet mistook the dark spot for an orifice. Osborne's figure shows that it is due to deeply stained nuclei in small cells closely packed. Colorless parenchyma surrounds a central shaft of stained cells, decreasing in color up the root.

With high power there are several groups of cells with pale pink, somewhat plasmolyzed cytoplasm. Their nuclei are crimson, and contain still darker nucleoli and chromatic granules.

Sidney Godolphin Osborne (1808–1889), clergyman and philanthropist of distinction, describes himself as a ''mere amateur'' in this research. ''I have made no attempt to resolve any question in chemistry.'' But by growing wheat seedlings in spring-water colored by carmine, vermilion, and indigo, he was prepared to demonstrate that ''the nuclei and the aggregations of formative matter which tend to nucleolar growth, will always be found to present a much deeper colour than the formative matter in the same cell with them'' (p. 114). ''It is impossible,'' he said, ''to value too highly the help given to

[15] Krause, in Wagner's Handwörterbuch of 1844 (Bd. 2, p. 119) had reported that dark granules, ''doubtless of silver chloride and reduced silver,'' were deposited along the margins of epidermal cells when treated with silver nitrate. F. Stadtmüller in his Historische Darstellung . . . der Silbermethode (Anat. Hefte, Arbeiten, 1921, Bd. 59, p. 79–210) does not include Hartig's observation, apparently preceded only by Krause's.

the study of cell-growth from the use of colouring matters applied to the growing plant'' (p. 113).

It was therefore no new method which Joseph von Gerlach (1820–1896) announced in 1858, prefaced by the statement that unless there were better visual aids, little was left to discover in human histology. By chance he had found that in animals color may spread from injected vessels into the surrounding tissue, just as Sarrabat, Reichel and others had long before seen in plants. His novelty had in fact become a topic of the day. Hermann Welcker, Privatdocent at Giessen, had published the year before, in Henle's Zeitschrift, 1857, that a frog's muscle, having one end immersed in red ink, drew up the color. Cross sections of the stained part showed the muscle corpuscles (nuclei) so surprisingly and brilliantly red that Welcker could hardly believe the tissue to be the same that he knew so well in the unstained state [16]. Lionel S. Beale, on issuing the 2nd edition of his ''Microscope'' in 1858, and still unaware of Hartig's and Osborne's publications, credited Welcker with the discovery of the carmine method, ''which has been employed of late years'' and is ''worthy of patient study.'' He thanks Doctor Harley for ''a beautiful specimen of the cells of the suprarenal capsule prepared in this manner.''

Though forestalled as a discoverer, Gerlach independently published in color a few effectively simple pictures showing what carmine could do with a Purkinje's cell and a Meissner's corpuscle. These were presumably the first figures in color of stained cells from the tissues of the higher animals. He advised the reader to put a section of cartilage, muscle, epithelium or connective tissue into an ammoniacal solution of carmine (carminsaures Ammoniak) and see for himself. That advice led to action.

It is a tedious and often heartbreaking task for an investigator to study an extensive literature only to find his independent discovery anticipated, perhaps in some obscure and forgotten

[16] Pages 230–231. The red ink (rothe Tinte) was a carmine solution, as assumed by Beale, and later reported to Gierke by Welcker himself (Gierke, 1884, p. 70). It contained potassium carbonate, tartar and alum; but ammonia, he thought, spoiled it.

paragraph. Gerlach spared himself that trouble, apparently not mentioning any literature of staining whatever. When, in 1859, he reported to the Erlangen Society "Ueber die Einwirkung von Farbstoff auf lebende Gewebe," his major finding was that living tissue did not stain. His tadpoles lived a month unaffected in the carmine-tinged water that stained dead ones. He did not cite Hartig's publication of 1854 (col. 576) that "staining does not occur before the cellular tissue is injured or functionally deranged: Algae, Chara, Lemna, Hydrocharis grow readily (freudig) in carmine solution with no staining of their nuclei." Nor did he recognize Cotta's emphatic statement that living bark and pith will not stain. Gerlach found that ciliated cells were not colored until the motion of their cilia had ceased. Five years earlier Hartig had said that bodies in the root-hairs of Hydrocharis stained, but not until the circulating sap had stagnated (col. 555).

Therefore one may question whether the "Einführung der Färbungsmethoden in die mikroskopische Technik" is rightly ascribed to Gerlach (as by Spalteholz, 1904) — whether Gerlach "should be, and generally is, considered the father of the technic of staining" (Conn, 1940). With keen discrimination the marble bust of Gerlach at his University of Erlangen is not inscribed — "Durch seine Carminfärbung ist daher Gerlach der Begründer der gesammten Tinctionstechnik geworden,"[17] but instead — *Tingendi arte innititur Histiologia* — "Histology depends on staining." From information at present available, though further reading of musty papers may well revise it[18], the staining of microscopic sec-

[17] "Joseph von Gerlach," in Allgemeine deutsche Biographie, herausgegeben durch die historisch Commission bei der Kön. Akad. d. Wiss., Bd. 49, p. 306. Leipzig, 1904.

[18] In Malpighi's De cerebri cortice, 1666, it is recommended that ink be applied to the shrunken and somewhat nodular surface of a boiled brain, which is then gently wiped with cotton, leaving ink in the furrows. No staining is involved, and generally no distinct pattern is revealed. A. W. Meyer in his "Rise of Embryology" ('39, p. 277) credits Cornelis Drebbel with the use of a stain, citing A. J. van der Aa's Biographisch Woordenboek. But neither there nor in G. Thiere's "Cornelis Drebbel, 1572–1633" (Amsterdam, 1932) is any biological use implied for the famous tin-mordanted cochineal that Drebbel introduced into the textile industry.

tions began with Leeuwenhoek, rather patronizingly called by the Fellows of the Royal Society, that "Curious and Inquisitive Person." But the saffron which he employed had already been used by Vieussens to inject the cerebral cortex, and find new support for an ancient error.

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CHANGES IN LUNG STRUCTURE DURING ASPIRATION OF AMNIOTIC FLUID AND DURING AIR-BREATHING AT BIRTH

W. H. WHITEHEAD, W. F. WINDLE[1] AND R. F. BECKER
Anatomical Laboratories, Northwestern University Medical School, Chicago, Illinois

TWO TEXT FIGURES AND ONE PLATE (FOUR FIGURES)

Whether or not the fetus normally aspirates amniotic fluid is a question which has been debated for many years. Some investigators have held that intrauterine respiratory movements are a normal phenomenon. Others, although recognizing that fetuses may execute such movements occasionally, have thought these movements to be abnormal, suggesting that they should be viewed with apprehension lest they lead to aspiration of the amniotic fluid.

The subject of intrauterine respiratory movements has been reviewed recently (Windle, '40, '41). Evidence available at present seems to indicate that fetuses are apneic during the greater part of gestation, and that fetuses whose oxygen requirements are adequately met are apneic until birth. Any condition leading to inadequate oxygenation may induce intrauterine respiratory movements.

Experimental injections of dyes or particulate matter into the amniotic sac (Wislocki, '20; Perry, '31; Snyder and Rosenfeld, '37; Benecke, '38; and Shock, '41) and studies of the vernix-filled lungs from still-born infants (Farber and Sweet, '31) have proved that aspiration of amniotic sac contents can occur. Evidence that aspiration does not occur normally in the guinea pig near term resulted from experi-

[1] Aided by a grant from the Women's Faculty Club of Northwestern University Medical School.

ments in which radio-opaque substances were introduced into the amniotic sacs of non-narcotized animals (Windle, Becker, Barth and Schulz, '39). So long as anoxial conditions were avoided, thorotrast injected into the amniotic fluid was not aspirated. When the oxygen level of the fetal blood was lowered or the carbon dioxide level raised and intrauterine

Fig. 1 Roentgenogram of a guinea pig fetus in utero on the sixty-third day of gestation. Thorotrast (0.8 cc.) was injected into the amniotic sac and the mother was rendered cyanotic with nitrogen containing a low concentration of oxygen. Intrauterine respiratory movements were observed. The bronchial tree contains the thorotrast but alveoli of the lungs do not.

Fig. 2 Roentgenogram of a still-born guinea pig on the sixty-fourth day of gestation. Thorotrast (1 cc.) was injected into the amniotic sac on the sixty-first day. Daily roentgenograms revealed no lung shadows. Intrauterine asphyxiation during labor induced marked aspiration, filling the lungs with thorotrast. Shadows of the stomach and intestines indicate fetal swallowing.

respiratory movements thus induced, the fetuses aspirated amniotic fluid into the trachea and bronchi but not into the alveoli. However, the lungs became filled with thorotrast during asphyxial death in utero. Two experiments are illustrated in figures 1 and 2. These observations are difficult to reconcile with the view that fetal respiration aids in structural

differentiation of the normal lung (Snyder and Rosenfeld, '37).

Differences in opinion would seem to depend upon lack of knowledge of the normal structural picture of the fetal lung at full term. Histologically the lung of the still-born human infant contains many fluid-filled alveoli and alveolar ducts (Adair and Potter, '39; Potter and Adair, '40). Similarly in experimental animals it has been observed that fluid-filled spaces occupy 20 to 30% of the lungs before breathing has commenced (Addison and How, '13). These observations suggest that a considerable quantity of fluid, coextensive with that in the amniotic cavity, is contained in the full-term fetal lung. They do not prove that this is the normal condition, because it is possible that during death of the infants in utero, and during the period preceding the experiments in animals, asphyxial conditions had induced intrauterine respiratory movements and led to aspiration. Indeed in the light of present knowledge it seems certain that this was the case and that the partially expanded, fluid-filled lungs in question do not present the true picture of apneic specimens. In the present study we aim to clarify the structural picture of the lung just before and after birth of the guinea pig.

METHOD

Sixteen full term guinea pigs (eight litters) were used. Five were prevented from breathing or aspirating by clamping the trachea at natural birth or at caesarean section performed either after local anesthesia of the abdomen or under nembutal narcosis. Four litter mates of the former were induced to perform respiratory movements and aspirate their amniotic fluid by clamping the uterine arteries and asphyxiating them. Aspiration of fluid was verified by direct observation. The remaining specimens breathed air for periods varying from 2 minutes to 9 hours. They were killed by clamping the trachea during inspiration.

The specimens were fixed by vascular perfusion with formalin and, after removing the skin of the chest, were immersed

in the fixing fluid until the lungs had become hardened in situ. The thoracic viscera were then removed in one piece, imbedded in nitrocellulose and sectioned in a plane at right angles to the tracheal axis. Representative regions were studied.

RESULTS

Study of the lungs of these experimental animals revealed three quite distinct conditions: initial atelectasis, partial expansion by amniotic fluid, and partial expansion by air.

The lungs of the guinea pigs which had no cause to gasp and aspirate fluid before birth and had not been allowed to breathe air were almost completely unexpanded. They were compact, gland-like organs whose vascular and lymphatic channels were well filled, but whose respiratory passages were not. Bronchioles, alveolar ducts and alveoli could be identified. Only the larger bronchioles appeared to have lumens of appreciable size. Folds of epithelium filled the smaller bronchioles (fig. 3), and the more distal passages were represented by epithelial masses which often had no visible free surfaces. The epithelium of respiratory bronchioles, alveolar ducts and many of the alveoli was columnar or cuboidal, but cell boundaries could not be discerned (fig. 4, a). Nuclei were spherical to ovoidal and stained darkly with hematoxylin. In general it was evident that the guinea pig lung at full term resembles an atypical compound tubulo-alveolar gland with very rich blood supply and lymph drainage. Few published illustrations of the full-term still-born lung demonstrate this condition. All show partially expanded alveoli; but Farber and Wilson ('33) recognized small regions of true fetal atelectasis in human new-born lungs and their figure 1 is similar to our own preparations.

The lungs of guinea pigs which were observed to aspirate amniotic fluid during asphyxiation in utero were found to be partly expanded (fig. 5). No longer did they resemble a gland. Bronchioles had opened and in their lumens masses of debris, apparently meconium, could be observed. This material extended into some of the respiratory bronchioles and alveolar

ducts. The epithelial linings of alveolar ducts and alveoli was not quite like that in the atelectatic specimens. In many places is was lower and the nuclei were flatter. There were still many regions of initial atelectasis in which the alveoli had not filled with amniotic fluid, and in them the epithelum was higher. Expansion with fluid was more marked in the apex and along the midventral diaphragmatic margin than elsewhere. The alveoli there occasionally approached the size of air-filled units.

The lungs of guinea pigs which had breathed air for several minutes to several hours showed varying degrees of expansion (fig. 6). However, many small regions retained the atelectatic condition, especially around veins, close to branching portions

TABLE 1

Percentage of space in guinea pig lungs before and after breathing.

INITIAL ATELECTASIS			ASPIRATION			AIR-BREATHING		
Specimen	Per cent	Average	Specimen	Per cent	Average	Specimen	Per cent	Average
A	3.7	4.7[1]	C	17.3	16.0	2 min.	28.0	
B	5.6		D	14.7		11 min.	35.6	34.4
						60 min.	39.7	

[1] When a correction is made for lymphatic and tissue spaces which were measured, this value is reduced to approximately 2%.

of the bronchial tree and in the mediastinal portions of the lungs. The minute structure of these regions was no different from that of the lungs from fetuses which had not been allowed to gasp in utero.

In order to express quantitatively the relation between solid pulmonary tissue and open spaces in the lungs under the three physiologic conditions of the present experiment, we traced ten representative fields from each of several histologic specimens with a camera lucida at a magnification of 730 times. The total area of each field and the sum of the area of the fluid- or air-filled spaces were computed with a planometer. Results of this study are recorded in table 1.

Although we found that about 5% of the atelectatic fetal lung at term was occupied by apparent spaces, it should be noted that lymph channels and other connective tissue spaces, often difficult to differentiate from alveolar spaces while making the camera lucida tracings, were included in all measurements. When a correction was made for these, the average amount of true lumen space in the atelectatic lungs was found to be less than 2% of the entire sectional area. Some regions lacked alveolar spaces completely. Lymph vessels seemed to be more compressed in the amniotic fluid-filled and the air-filled lungs. Consequently the values obtained by planometer in these specimens more nearly represented the true relationship between lumens and solid portions.

DISCUSSION

The present experiments reveal that the lung of the guinea pig at full term is compact. This initial atelectasis indicates that no fluid had been aspirated by action of intrauterine respiratory movements. The alveolar epithelium was largely cuboidal and was not flattened as would be expected had previously expanded alveoli become collapsed by absorption of the contained fluid. The difference between initial atelectasis and resorption atelectasis was well shown by Farber and Wilson ('33). Intrauterine respiratory movements have not been observed in pregnant guinea pigs under normal conditions, but have been induced by raising the carbon dioxide level or lowering the oxygen level of the blood (Windle, Becker, Barth and Schulz, '39). This fact together with the demonstration of initial atelectasis leads to the conclusions either that intrauterine respiratory movements do not occur normally in this animal or that they are too weak to cause aspiration. This raises a doubt that they are a normal phenomenon of significance in other species including man.

That initial atelectasis prevails in the lung alveoli of human fetuses until birth seems probable. Lungs of still-born infants resemble those of guinea pigs which were deliberately asphyxiated and thus induced to aspirate the amniotic con-

tents. However, small pulmonary regions showing initial atelectasis have been found in still-born lungs and in the lungs of infants dying during the early neonatal period (Farber and Wilson, '33). We too observed such regions in the guinea pigs which had died asphyxial deaths in utero as well as in those which had breathed air for a short time.

If the fetus does not aspirate its amniotic fluid normally, one can scarcely believe that absorption of amniotic fluid by the fetal pulmonary epithelium is a normal phenomenon (Wislocki, '20). The potential spaces of the alveoli and alveolar ducts connect with the amniotic cavity by way of bronchioles, bronchi and trachea, and at least the larger tubes contain fluid coextensive with that in the amnion. Is there normally a movement of this fluid and is it inward or outward? The observations of Potter and Bohlender ('41) in two still-born human infants in which all or part of the alveoli lacked connections with the amniotic sac because of congenital occlusions of bronchus or trachea suggest to us that a flow may occur outward, although not so stated by them. These investigators found the alveoli rather well expanded. The source of the fluid which produced this expansion can only have been the tissues and blood of the fetus itself. The mechanism which provides a film of fluid on alveolar surfaces in the adult may be operating in the fetus. It would seem that there is a gentle flow of transudate from atelectatic fetal alveoli toward the mouth and amniotic cavity. This could be of some importance in keeping the respiratory passages free of heavy mucus, vernix and other debris, and thus assure that the bronchi are not occluded by these substances at the time of the first inspiration of air at birth. May it not be that the lung thus contributes to the amniotic liquid rather than that it aids in absorption of it?

In the present study we were not directly concerned with the minute structure of pulmonary alveoli, and there is no need to review the literature on this subject at present. It has become apparent to us that the nature of alveolar epithelium should be reinvestigated from the standpoint of its develop-

ment. Even in the most recent developmental studies, normal physiologic conditions did not prevail in late fetal life and inadequate interpretations of histologic observations resulted (Ham and Baldwin, '41; Norris, Kochenderfer and Tyson, '41). It has not been generally appreciated that atelectasis persists until birth. The abnormal changes in alveolar epithelium during aspiration, premature breathing, and especially during deliberate artificial inflation of the still-born lung, distorts the developmental picture considerably. Bensley and Groff ('35) observed that cuboidal epithelium persists as a lining of the pulmonary alveoli of the rat as late as the day before birth and that it is stretched into a thin membrane with successive inspirations just before and after birth. These observations are in accord with ours in as much as we observed a cuboidal type of lining in the alveoli during initial atelectasis of the full-term guinea pig but found thinning of the epithelium in those which had aspirated. Normally the cuboidal epithelium would seem to persist until air breathing is initiated, and it is probable that the thinning of alveolar epithelium comes about promptly during the early neonatal period.

CONCLUSIONS

1. Intrauterine respiratory movements of sufficient strength to cause aspiration of amniotic fluid do not occur as a normal physiologic phenomenon in the guinea pig.

2. Initial atelectasis of the fetal lung persists until birth of the guinea pig. The lungs are solid and gland-like organs in which alveolar spaces comprise only about 2% of the entire mass.

3. The lungs of the fetal guinea pig expand partially and unevenly when asphyxia induces intrauterine respiration of sufficient strength to cause aspiration of amniotic fluid. The amount of alveolar space is increased to approximately 16% of the entire pulmonary mass.

4. With the advent of air-breathing, the lungs undergo a gradual expansion. By the end of an hour, alveolar spaces occupy about 40% of the lungs. One to 9 hours after birth

small regions of initial atelectasis still persist throughout the lungs.

5. The alveoli and alveolar ducts of the guinea pig lungs at full term appear to be lined almost completely with cuboidal epithelium, but in most of these units, lumens are extremely small, often invisible.

6. It is suggested that the small quantity of fluid contained in the fetal trachea and bronchi is not of amniotic origin, but is a transudate formed in the lungs. It is suggested, furthermore, that this flows gently outward to the amniotic space, serving to prevent mucus, vernix and other debris from settling in the respiratory passages and occluding them at the time of the initial inspiration.

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PLATE 1

EXPLANATION OF FIGURES

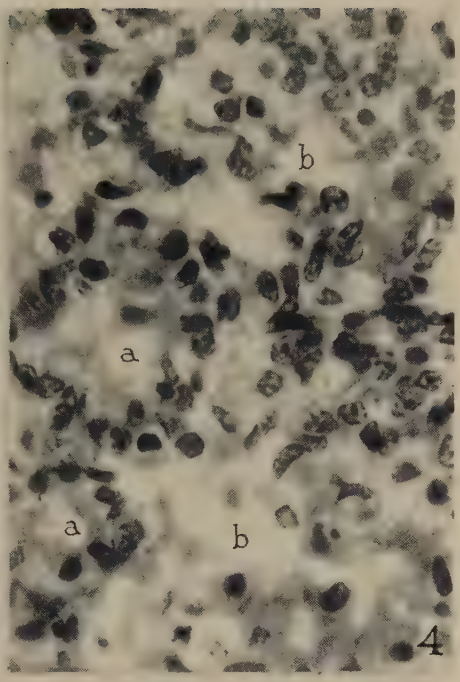
3 The lung of an apneic guinea pig at full term showing initial atelectasis. Respiratory bronchioles, alveolar ducts and alveoli are unexpanded. Most bronchioles are unexpanded, like the one in this field. × 80.

4 Initial atelectasis; a, alveoli or alveolar ducts; b, tissue spaces. × 760.

5 Aspiration of amniotic fluid caused by asphyxia has produced partial expansion of bronchioles, respiratory bronchioles, alveolar ducts and, to a less extent, alveoli. × 80.

6 Lung of normally delivered guinea pig after 11 minutes of air-breathing. Note the open respiratory bronchioles and alveolar ducts with many alveoli. Regions of initial atelectasis give no indication of previous expansion from aspiration of amniotic fluid. × 80.

3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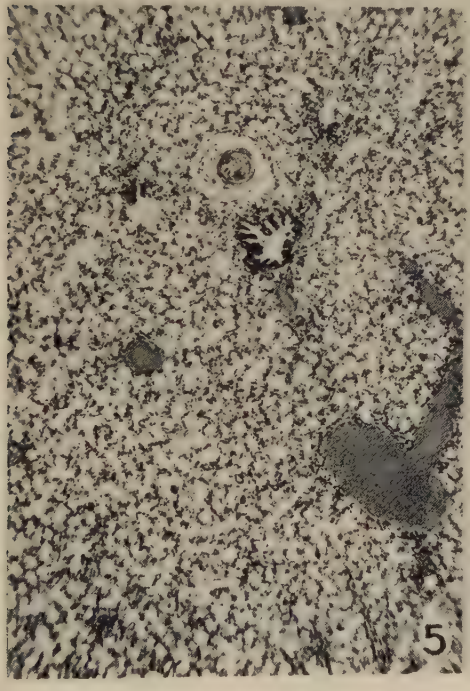
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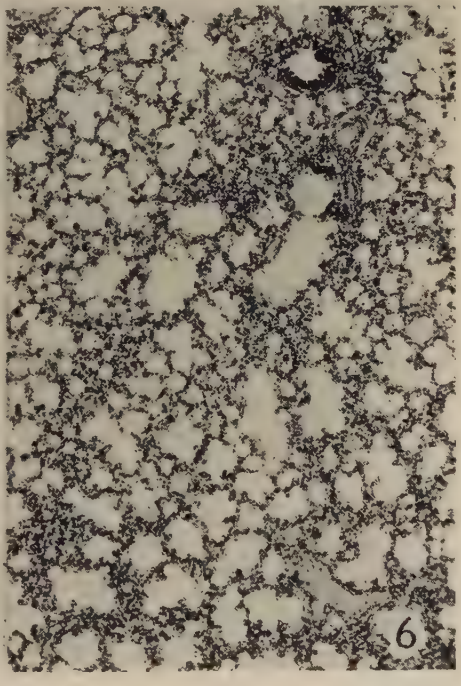
6

EARLY HUMAN TWINS WITH PECULIAR RELATIONS TO EACH OTHER AND THE CHORION[1]

PETER GRUENWALD
Department of Anatomy, The Chicago Medical School, Illinois

TWO TEXT FIGURES AND TWO PLATES (SIX FIGURES)

In accordance with the comparatively small number of early human embryos on record, only very few instances of twins in human ova of the first month have been described. Apart from the interest commanded by this scarcity of related findings, the twins to be described here are of importance because they exhibit a type of mutual relationship which, to the best of the author's knowledge, has never been described before. These peculiar relations are probably the cause of a malformation of one of the partners, ourentery (Rabaud, '00), which has never been seen in man. Furthermore, these twins represent, as will be pointed out in the course of the present discussion, an early stage of acardius formation.

DESCRIPTION OF THE SPECIMEN

The twins were found in an ovum which had been fixed in Bouin's fluid, sectioned serially at 10 μ, and stained with hemalum and eosin. The embryos proved to be cut transversely, and a sagittal reconstruction was made in which the inner surface of the chorion nearest the dorsal sides of the embryos was arbitrarily taken as a straight line (fig. 1). The principal features will be described with the help of this reconstruction. The embryo directly attached to the chorion

[1] A brief description of these twins was presented at the Fifty-seventh Annual Meeting of the American Association of Anatomists (see Anat. Rec., vol. 79, suppl., pp. 27–28, 1941).

will be referred to as twin A, the other one as twin B. It will be noticed that twin A is incompletely preserved. Its cranial half is torn off and missing; it must have escaped through a small tear in the chorion. Normally developed organs of both twins will not be described unless such description is necessary for the following discussion.

The incompletely preserved embryo A will be considered first. Figure 1 shows the extent of the artificial defect. The missing portion comprised the cranial part of the embryo and extended to a point just beyond the level of the vitelline duct. The persisting portion in the series is normally developed (figs. 7, 8). It is attached to the chorion by a normal body stalk containing the narrow allantois and the umbilical vessels, and covered on its dorsal side by amniotic epithelium (fig. 8). A tapering extension of the amniotic sac follows the body stalk almost to the chorion; the allantois ends at a slightly shorter distance from the embryo (fig. 1). In the body itself, only the neural primordium must be mentioned. It does not form a closed tube in the preserved portion, in contrast to that of the other twin which is almost completely closed. It will be shown later that the condition found in twin A is probably the normal one. The umbilical veins of embryo A as shown in figure 7, are of interest because they lead not only to that embryo but, through large anastomoses, also to the umbilical veins of twin B as will be pointed out soon in more detail.

It can be assumed that the missing part of embryo A had escaped through an opening of the amniotic cavity on its ventral side, between twin B and the preserved part of twin A. A small defect (not shown in fig. 1) in the wall of the yolk sac of embryo B probably indicates the former insertion of the vitelline duct of twin A, although the possibility cannot be definitely excluded that both twins had separate yolk sacs (see below).

Twin B is normally developed in its cranial parts, approximately to the level of the liver primordium (figs. 1, 3, 4). No vitelline duct has been formed in this embryo, due to the absence of a detached hind-gut. Instead, the part of the body

normally containing the hind-gut is invaginated into the yolk sac and covered with entoderm in such a manner that its ventral surface is lined by what would normally be the dorsal wall of the gut (fig. 5). The structures dorsal to the gut, including neural tube, notochord and somites, show normal mutual relations. There is, however, no normal transition of the neural tube into an open groove and then a trunk-tail-node,

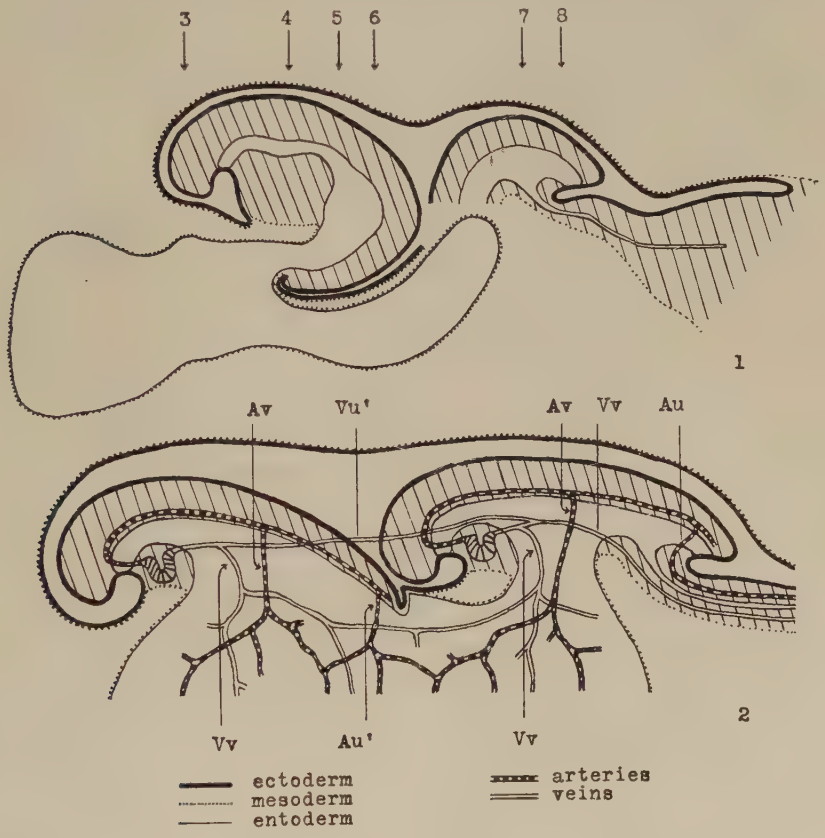

Fig. 1 Graphic mid-sagittal reconstruction of the twins, × 20. Twin B to the left, the preserved part of twin A to the right. The arrows indicate the levels of the figures with corresponding numbers.

Fig. 2 Diagram of a probable early stage of the same twins, also showing their principal blood vessels. Only part of the yolk sac is shown.

No ectodermal or mesodermal intraembryonic organs are shown in these figures.

as characteristic of this stage. The neural primordium forms a tube almost to the caudal tip of the body, with only a small opening near its end. No normal trunk-tail-node is present. The epidermal covering of this part of the body forms, together with the adjacent amnion, an ectodermal tube appearing sickle-shaped on cross section (fig. 5). The mesodermal surfaces of amnion and yolk sac are closely applied to each other in this area. This condition represents typical ourentery as described first by Rabaud ('00) in chick embryos. In the cases presented by Rabaud, only a shorter caudal part of the body was affected, and consequently the cranial part of the hind-gut was present as well as the allantois; the structures normally composing the tail were invaginated into the proximal part of the allantois. No cases of this malformation were ever described as such in other groups of amniotes; however, as pointed out previously (Gruenwald, '41), a case of "Dottersackbruch" found in a lizard embryo by Peter ('34) actually exhibits this anomaly. In man, the present case is the first on record, apart from small protrusions of axial organs into the hind-gut in otherwise severely maldeveloped embryos, which might be regarded as very low degrees of the malformation in question. Body stalk, allantois, and cloacal membrane are absent in the present case (twin B).

The lack of any direct attachment of twin B to the chorion raises the question where this embryo had the connections with extraembryonic parts necessary for its metabolism. Figure 1 shows that the only structure connecting twin B with other parts of the ovum, is the amnion which is common to both twins. Since twin B is completely preserved, it can be assumed with certainty that its body had no direct connection with that of twin A. There is, however, a strong possibility that a common yolk sac had provided for another communication, more important even than that through the amnion. Due to the damaged condition of twin A, the relations of the yolk sac cannot be established with certainty. Blood vessels leading out of twin B or into it, were found in two areas only. One of these was mentioned above when it was stated that the

vessels occupying the position of normal umbilical veins in twin A, continued to twin B. They can be traced from their normal position in twin A, in the body wall near the attachment of the amnion to a similar location in the lateral body wall of twin B (fig. 5). No details of their course from one twin to the other can be given because the respective area is damaged by the artificial defect of twin A. However, the continuity can be traced beyond doubt through the amnion. No corresponding arteries were found. The second area in which blood vessels enter or leave embryo B, is its attachment to the yolk sac. No exact survey of these vessels could be made because of the abnormally extensive and close relations of the embryo to the yolk sac; it must be emphasized, however, that this is the only area where arteries lead out of twin B. This fact makes the assumption of a yolk sac common to both twins highly probable. The circulation in the wall of this common sac would then have provided for the arterial limb of the circulation, the venous part of which consisted of the above mentioned umbilical veins, and also veins of the yolk sac.

An attempt was made at determining the age of the twins. Thirteen pairs of somites were counted in the completely preserved twin B. This, however, may not indicate the correct stage of general development because of the malformations present in the caudal part of twin B. The absence of a typical posterior neuropore and, caudal to it, a neural plate passing over into a trunk-tail-node, points to an anomaly in the differentiation of the early embryonic organs in that region. The corresponding portion of twin A appears to be normally developed (figs. 7, 8). This region, as well as the apparently normal cranial part of twin B, were compared with Politzer's ('28) detailed description of a normal 18-somite human embryo. Particularly the nervous system of that normal embryo as also described by Sternberg ('27), was used for comparison. The corresponding parts of the twins show slightly earlier stages of development. The anterior neuropore of twin B, for instance, is 0.23 mm. long against 0.1 mm. in the 18-somite embryo. The condition of the ventral body wall and its reflection

into the amnion likewise presents a slightly earlier condition in the twins. It can therefore be concluded that the twins were fixed in a stage of between 13 and 18 pairs of somites.

DISCUSSION AND CONCLUSIONS

In view of the damaged condition of twin A an attempt must be made at reconstructing the relations of this embryo to twin B, as well as the probable condition of its cranial part. It was pointed out in the preceding section of this report that both twins probably had a common yolk sac. Conclusive evidence of the existence of twins with one common yolk sac has been shown by Arey ('22) in an ovum containing two normal embryos attached by individual vitelline ducts to one yolk sac. Another more complicated case briefly described by Heaney and Bartelmez ('31), will be referred to later in this discussion. The diagram given in figure 2, showing a probable early stage of the twins described in the present report, is therefore based on the assumption of a yolk sac common to both embryos. This same diagram, as well as the reconstruction (fig. 1), shows both twins with their midplanes coinciding, and both embryos facing in the same direction. Direct evidence of this is shown, in spite of the damage to twin A, by the condition of the amnion and the umbilical veins running from each side of twin B to the same side of twin A. As was mentioned in the introduction, this peculiar relation of twins is so far unique. To the best of the author's knowledge the two partners face in opposite directions in all cases of twins or double monsters with coinciding midplanes described up to the present time.

The mutual relations of the twins have a direct bearing on the question of the probable condition of twin A's head. The inversion of the caudal part of twin B was probably brought about by mechanical interference with the head of twin A (fig. 2), and the possibility must be considered that similar pressure may have been exerted in the opposite direction as well, causing inversion of the head of twin A. In bird embryos where inversion of one end of the body is by far more fre-

quent than in mammals, omphalocephaly (inversion of the head) occurs more often than ourentery. Its causation in single embryos is not purely mechanical (Gruenwald, '41); in double monsters, however, omphalocephaly is very probably due to mutual interference of the partners (Gruenwald, '37, '41). This experience is in favor of the possibility that the head of twin A was affected with omphalocephaly. On the other hand, an omphalocephalic head torn away from the yolk sac by force, could hardly have caused so little damage to the yolk sac as was actually found. For this reason the alternative of a normally developed head is considered more probable and was followed in preparing the diagram of figure 2.

According to the present findings and considerations, the following appears to be the most probable formal genesis of the malformation in question. Two embryos developed in one embryoblast in such a manner that they had common amnion and yolk sac. Their primitive streaks were situated approximately in one straight line and both oriented in the same direction. Consequently, the caudal end of only one of them (twin A) could establish normal relations to the chorion by means of a body stalk, whereas the caudal end of the other twin (B) was situated near the cranial end of A but nowhere near the chorion. Whether the absence of structures corresponding to body stalk and allantois in twin B is a sequel of these abnormal relations, or a primary defect of the region near the caudal end of the primitive streak, cannot be established. The absence of a cloacal membrane and a trunk-tail-node of normal dimensions points toward the latter alternative, but both causes may well be jointly responsible for the development of the defect. Figure 2 shows diagrammatically how mechanical interference of the two twins probably caused ourentery in twin B, in a similar manner as pointed out previously ('37) in a discussion of omphalocephalic twins in birds. This depression of the caudal end of the body into the yolk sac must have occurred at an early stage, before the appearance of a distinct tail fold could assure normal detachment of the trunk-tail-node region from the blastoderm.

As far as could be ascertained, twin A has a normal vascular system (fig. 2). The cranial part of twin B's vascular apparatus including the vitelline vessels, is also normally developed. There is, however, a serious disturbance in the development of its umbilical vessels, as is to be expected in accordance with the defect of body stalk and allantois. Whether or not any arteries found in the location indicated by Au′ in figure 2, are to be considered as modified umbilical arteries, will not be discussed. For the circulation itself this question is unimportant since these vessels, just as the vitelline arteries, branch in the wall of the yolk sac. Umbilical veins were found to be present, connected indirectly with the circulation of the chorion by their anastomoses with the corresponding veins of twin A. Due to these peculiarities, blood leaving the body of twin B could go only through the vascular network of the yolk sac and then either back to twin B or, through anastomoses in the wall of the yolk sac, to twin A and thus indirectly to the chorion. Blood returning to twin B could flow either through the umbilical veins, or again through anastomoses in the yolk sac circulation (fig. 2). This shows that twin B essentially depended in its circulation on twin A; its blood had to pass through the other twin's heart at least once on its way to the chorion and back. Somewhat similar conditions were found in a pair of human twins by Heaney and Bartelmez ('31). In that case, one normal embryo was found occupying the chorion together with a smaller, apparently underdeveloped twin. The embryos had separate amnions, but the smaller one had no umbilical cord. Its only connection with its surroundings consisted of a group of blood vessels leading to a yolk sac which was otherwise in normal topographic relations to the larger twin. In that case the entire circulation of the smaller embryo must have gone to the chorion via yolk sac and body of the other twin.

These two cases can safely be regarded as early stages of acardius formation although it cannot be asserted that in any of them the "dependent" twin would have developed into a typical acardius as far as its body is concerned. In a sum-

marizing review of the subject Schwalbe ('07) cites two contrasting views of the origin of acardiac twins, and there has been no change in the status of the problem since his report. One of these views holds that the acardius is a primarily maldeveloped twin whereas, according to the other opinion, an anatomically normal embryo becomes an acardius if the propelling power of its heart is inferior to that of the other twin. The present observation, as well as that of Heaney and Bartelmez, suggests a third possibility, namely, abnormal topographic relations of a twin with a normally developed body. (The additional malformation in the present case is only incidental to the vascular condition.) The present author believes, however, that no decision is to be made among these possibilities. Any anomaly, anatomical or functional, intraembryonic or extraembryonic, may turn one twin into an acardius if its circulation becomes dependent on that of the other embryo. There is no reason to assume that all acardii must owe their development to one and the same of the above mentioned three possibilities.

SUMMARY

1. A serially sectioned human ovum is described, containing twins with unusual malformations and mutual relations. The embryos are in a stage of between 13 and 18 pairs of somites.

2. The original longitudinal axes of both embryos are in one line, with both twins facing in the same direction. In all other known twins or double monsters with coinciding axes in vertebrates, the partners face in opposite directions.

3. Only twin A shows a normal attachment to the chorion. Body stalk and allantois are absent in twin B. This embryo is not directly attached to the chorion; it is connected only with twin A by the common amnion and probably a common yolk sac. Its umbilical veins lead through the amnion into those of twin A; umbilical arteries are absent.

4. Blood of twin B must have passed through twin A in order to reach the chorion. This shows that an early stage of acardius formation exists in the present twins. With regard

to the general question of the origin of that malformation, it is suggested that different anatomical or functional anomalies may lead to the type of circulation characteristic of an acardius.

5. Twin B is the first known case of typical ourentery in a mammalian embryo. This malformation, more common in birds, consists of an inversion of detachment of the caudal part of the body, so that it protrudes into the yolk sac and is covered by entoderm of the prospective hid-gut. In the present case the malformation was probably caused by pressure exerted by the other twin.

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PLATE 1

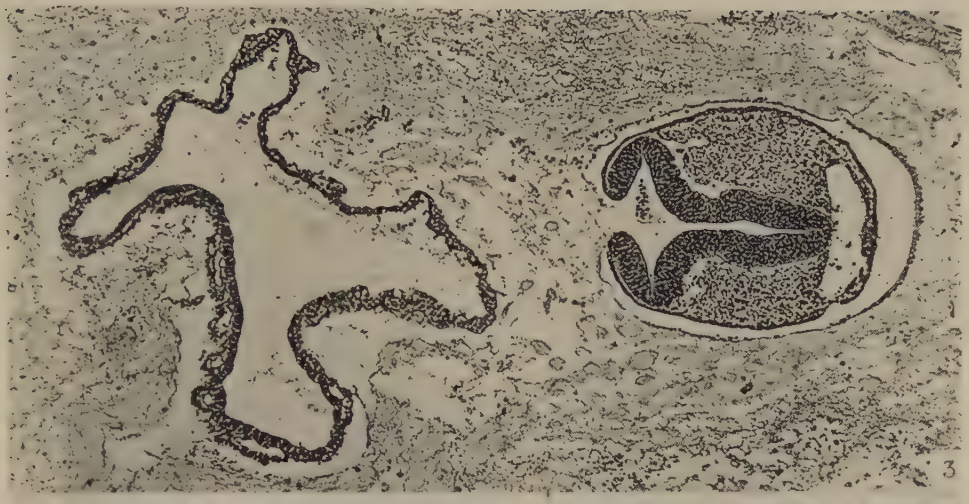
EXPLANATION OF FIGURES

3 Section of the normal head of twin B and the yolk sac.

4 Section of twin B at the level of the heart, and the yolk sac. The latter contains the ourenteric caudal end of the body.

5 Section of twin B, a short distance caudal of the anterior intestinal portal. The yolk sac, connected with the gut, contains the ourenteric part of the body. An arrow points to the right umbilical vein.

The levels of these sections are indicated in the graphic reconstruction (fig. 1). x amniotic cavity surrounding the ourenteric caudal portion of the body. × 62.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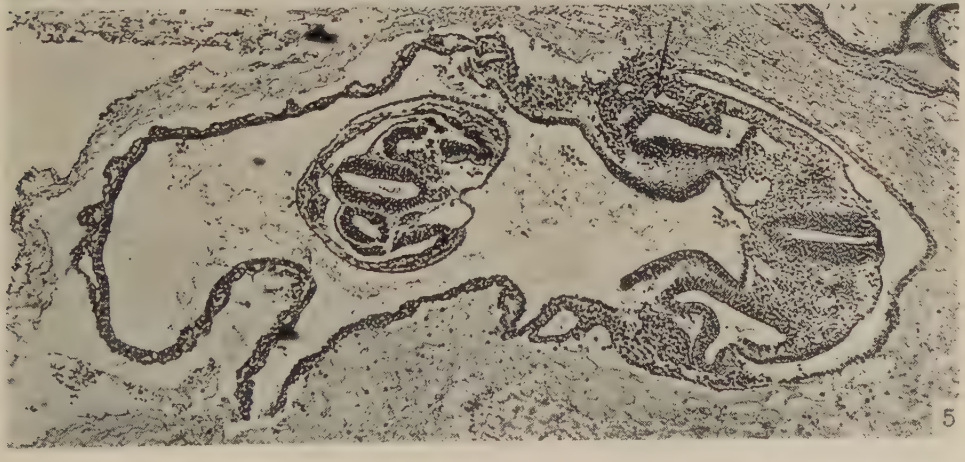
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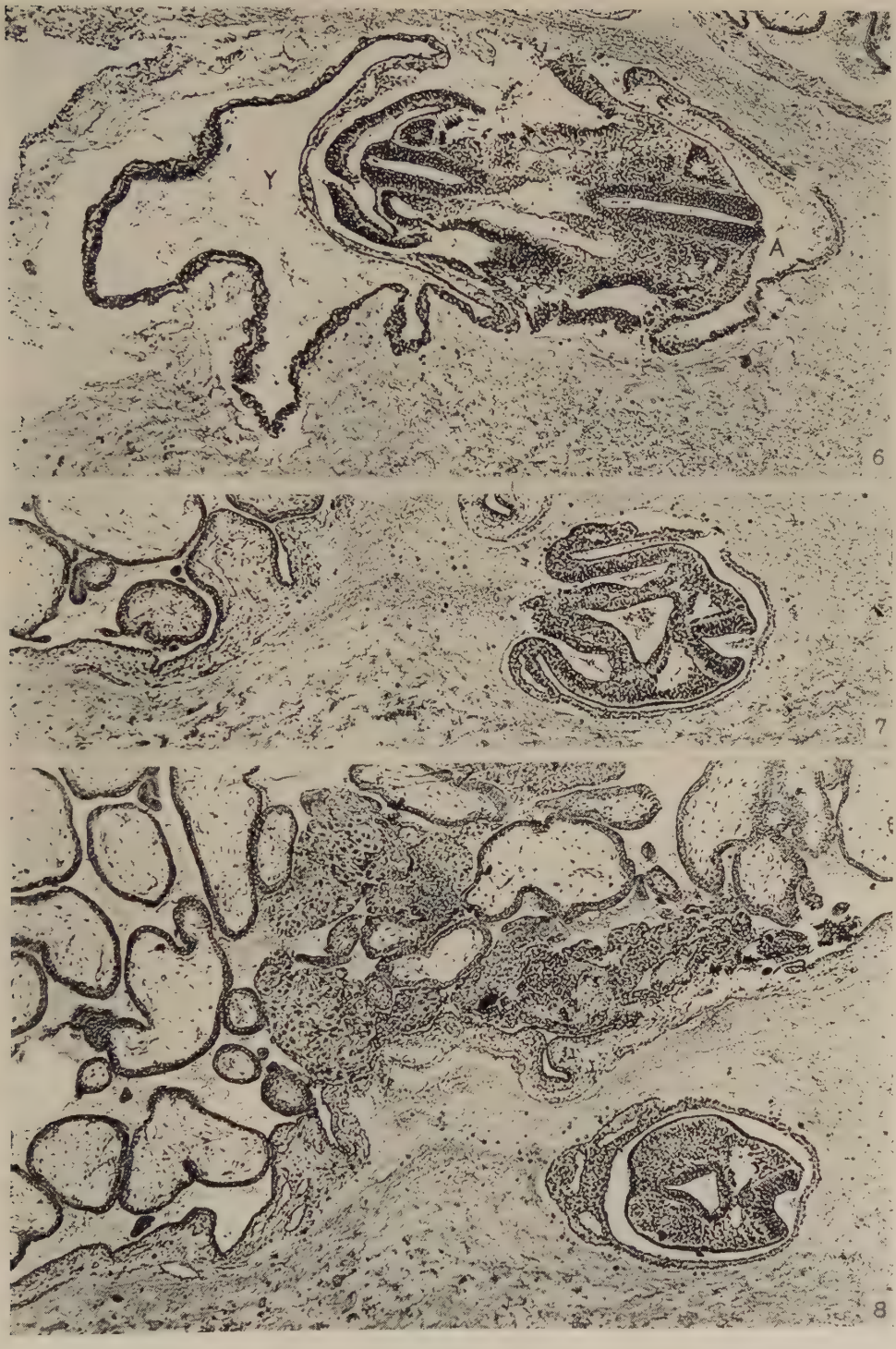
PLATE 2

EXPLANATION OF FIGURES

6 Section of twin B at the level of the transition from the normal to the ourenteric part of the body. A, amniotic cavity; Y, yolk sac.

7 and 8 Sections of the normally developed caudal portion of twin A.

The levels of these sections are indicated in the graphic reconstruction (fig. 1). × 62.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THE ORIGIN OF BINUCLEATE AND LARGE MONO NUCLEATE CELLS IN THE LIVER OF THE RAT [1]

H. W. BEAMS AND R. L. KING
Department of Zoology, State University of Iowa, Iowa City

ONE TEXT FIGURE AND TWO PLATES (TWENTY-THREE FIGURES)

INTRODUCTION

It has long been known to students of the cytology and histology of the mammalian liver that the nuclei of hepatic cells vary greatly in size and that in a certain percentage of cells a binucleate condition exists. Many efforts to account for this wide variation in size and number of nuclei among the hepatic cells have been made: some investigators holding it to be due to amitosis of the nucleus only (resulting in a binucleate condition) followed sometimes by a subsequent fusion of the daughter nuclei resulting in a single nucleus of large size. Others contend that the binucleate condition arises by a failure of the cytoplasm to divide at the time of karyokinesis ("cytoplasmic lag").

The want of dividing cells in the normal mammalian liver has no doubt contributed largely to the lack of crucial observations for a complete solution of these problems. We were stimulated to undertake this study because of a paper by Higgins and Anderson ('31) who showed, in addition to reviewing the literature on liver regeneration, that surgical removal of as much as 65 to 75% of the hepatic parenchyma was followed immediately by compensatory hyperplasia, resulting in a complete restoration by weight of the liver within 28 days. Such livers were found to be functionally normal. With this information at hand it was thought that a cyto-

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logical study of restoration livers might offer a clue to the mechanism by which the nuclei of hepatic cells are found to vary in size and number.

MATERIALS AND METHODS

White rats of the Wistar strain weighing approximately 100 gm. were used for this study.[2] In order to stimulate mitosis of the hepatic cells the median and left lateral lobes of the liver were removed, the procedure being that described by Higgins and Anderson ('31). The extirpated lobes were fixed immediately and served as controls in this study. Most of the liver from which our experimental studies were made was taken from rats that had been operated 3 days previously. The tissue was fixed in Bouin's solution and stained in Heidenhain's hematoxylin and eosin, or in Feulgen's solution counterstained in fast green.

DESCRIPTION

The normal cytology of the liver under different physiological conditions has been described by Mann ('32) and need not be repeated here. Suffice to say that in spite of the numerous functions of the liver there is no cytological evidence to show that specialized groups of hepatic cells exist, but that generally speaking all the hepatic cells appear similar, save for variation in size, number and form of mitochondria, number of nuclei, and amount of inclusion materials present.

In the hepatic cells of the normal liver of the rat the nucleus is usually centrally located and the cytoplasm appears coarsely granular, probably due in part to the inclusions present (fat, glycogen, and mitochondria). Whereas the uninucleate condition is predominant, binucleate cells are not uncommon (about 16% in rats, Münzer, '23). Examination of sectioned and stained liver from animals operated 3 days previously shows many cells in the process of division (figs. 11 to 24). In addition, mononucleated and binucleated cells with

[2] We wish to thank Prof. Emil Witschi for supplying us with the rats used in this study.

nuclei of different size are also observed. Figure 2 and the left cell in figure 4 show nuclei possessing the apparent normal diploid complement of chromatin, while the right cell in figure 4 and figures 8 and 9 show some multiple of the normal diploid chromatin content, probably a tetraploid condition.

Binucleate cells with the apparent normal chromosome complement in each nucleus are shown in figure 3 and figure 7. This will be obvious by comparing the nuclear size in figure 2 with that of figure 3. The volume of the nuclei in these particular cells is approximately the same. Binucleate cells with an apparent tetraploid chromatin content are observed in figures 5 and 6. Here the volume of each nucleus is approximately the same as that in the tetraploid mononucleated cells shown in figures 8 and 9.

In addition to diversity in nuclear size of "resting" cells, variation was also observed in chromosome numbers of dividing cells. For instance, figures 14 to 18 are various stages in mitosis of hepatic cells possessing the apparent "normal" diploid chromosome complement. While it was not possible to count accurately the chromosomes in these cells the size of their mitotic figures when compared to that of certain larger ones seems sufficient evidence for this conclusion. Polyploid cells are seen in mitosis in figures 19 to 24 (compare with figures 14 to 18).

Mitosis in mononucleate cells is typical with centrioles, spindle fibers, and the usual chromosome distribution. Cytokinesis takes place by furrowing at the stage shown in figure 18, resulting in two daughter cells. Definite cell membranes are demonstrated in our material, a fact constrasting with the descriptions of hepatic cells by certain other writers (Schafer, '29).

The mechanism by which diploid hepatic cells become polyploid may be illustrated in figure 20. Here, in spite of a normal karyokinetic mechanism, cytokinesis apparently did not occur, resulting in a binucleate cell with the two daughter nuclei still slightly elongated, which is characteristic of re-

cently divided nuclei. Furthermore, cells that have just completed karyokinesis or mitosis can be easily identified in hematoxylin and eosin preparations by their finely granular and pinkish staining cytoplasm. In this connection it should be stated that in this study we have not observed any evidence of amitosis either of the nucleus only, or of the cell as a whole.

Binucleate cells may undergo division in one of the following ways: (1) The nuclei enter prophase at the same time (figs. 10 and 11) giving rise to two sets of diploid chromosomes (fig. 19) which fuse at metaphase to form one spindle (fig. 22), complete division gives rise to two cells with large nuclei that are tetraploid (figs. 8 and 9). (2) The same karyokinetic phenomena may take place as in (1) except that cytokinesis does not occur (fig. 24), this results in a binucleate cell with each of the nuclei possessing the tetraploid chromosome complement (figs. 5 and 6). Just how many times the doubling of chromosomes can occur is undetermined but the statistical data indicate that some cells are probably of at least the 16-ploid condition. It is obvious from the cells illustrated here that doubling of the chromosomes is associated with a marked increase in size of the cell (compare fig. 2 with 3, figs. 8 and 9 with 5 and 6).

Mitotic divisions of cells other than hepatic (fig. 13) were also seen in our material. Fishback ('29) claims that in addition to division of hepatic cells, buds from the bile duct give rise to new hepatic cells which aid in the restoration of the hepatic parenchyma.

From a cytological study of this type, it was evident that variation in nuclear size of hepatic cells exists. However, it soon became apparent that little substantial evidence could be obtained by this method alone as to the probable number of categories into which the hepatic cells could be placed as regards their nuclear volume. Efforts to use the nucleoli, as has been done in certain other material to determine the chromosome number was found unreliable for the hepatic cells. It was, therefore, necessary to rely upon statistical methods for this part of the study.

MEASUREMENTS OF NUCLEI

The material used for measurement of nuclei consisted of that part of a liver removed during an operation, called here the control liver, and the restoring liver from the same animal removed when the rat was killed 3 days later (when mitoses, according to Higgins and Anderson ('31), are particularly plentiful). The tissue was fixed in Bouin's fluid, sections were cut in paraffin, and stained by the Feulgen method, counterstained with fast green. "Resting" nuclei of liver cells were drawn with a camera lucida at table level at a magnification of approximately 1900 diameters and measured to the nearest $\frac{1}{2}$ mm. The error in measuring is relatively slight compared with the difficulty of accurate drawing with a camera lucida; these two factors operate to mask any great variability of the material, so that differences which emerge are all the more significant. Personal errors are random. The nuclei of 200 "resting" uninucleate cells, selected systematically at random from the control liver were drawn, and the largest and shortest diameter measured and averaged. The two diameters were usually very nearly the same; deviations usually seemed to be due to drawing, because the nuclei are nearly spherical. The average diameters were then plotted, giving the distribution shown by the solid line in figure 1, above. The curve is bimodal with modes at 13.5 mm. and 17.0 mm. (over twice as many nuclei at the second as the first). Since the volumes of similar solids are proportional to the cube of a linear dimension, we have the proportion 2460:4913 so that the volumes are approximately 1:2.

Likewise the nuclei of 100 binucleate cells were drawn and measured. Their average diameters were then plotted, giving the distribution shown by the broken line in figure 1, above. The curve is likewise bimodal but with a significant difference; most of the nuclei here are at the first mode, fewer at the second. Since the two nuclei of a binucleate cell are similar in size ($r = +0.90 \pm 0.01$), if we plotted them according to nuclear volume these cells would coincide with the second mode of uninucleate cells. The second mode of bi-

nucleate cells would likewise be moved higher on the scale. There are no binucleate cells with nuclear volume equal to that of the smaller uninucleate cells. This is in agreement with the hypothesis that the binucleate cells are tetraploid or octoploid, the result of suppression of cytokinesis in one or two mitotic cycles respectively. The fact that the two

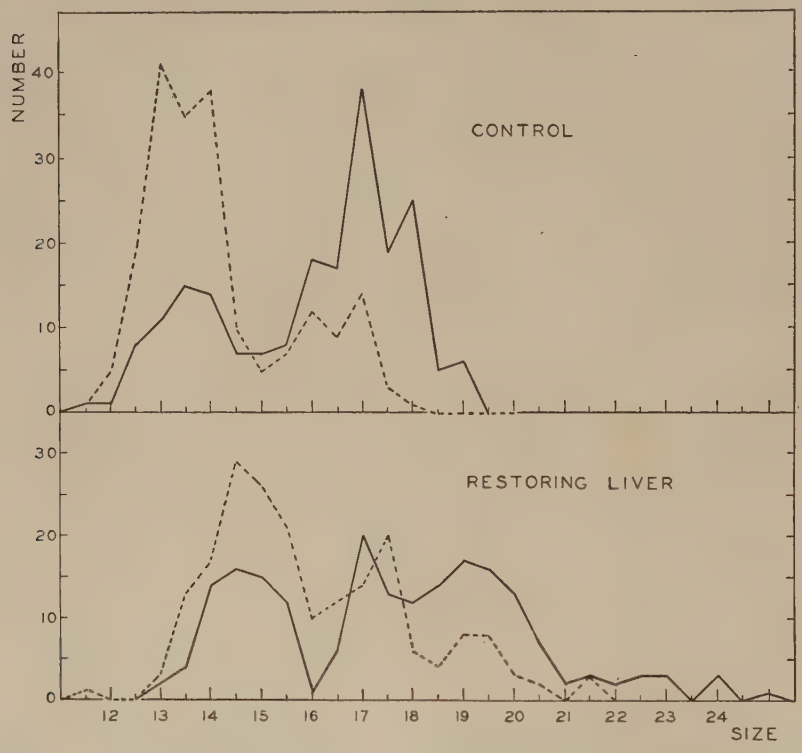

Fig. 1 Graphs of the distribution of nuclei arranged according to linear dimensions. Nuclei of uninucleate cells represented by solid line, those of binucleate cells by broken line. Control liver above, restoring liver below.

nuclei in a binucleate cell are so similar also points to their origin by karyokinesis. A count of 500 cells in the control liver gave $20.8 \pm 1.2\%$ binucleate cells.

A similar procedure involving a like number of cells was carried out with the restoring liver. The results appear in the graph, figure 1, below. The curves are bimodal but with quite

a lateral spread in the region of the second mode and a number of markedly large cells beyond the curve proper. The first mode is at 14.5 mm., the second would be at 18 or 18.5, the volume ratios would again be approximately 1:2 for both uninucleate and binucleate cells. The nuclei of the restoring liver seem to be larger than those of control; for binucleate cells the results of measurements are: control:

$M = 14.2 \pm 0.07$ mm., $\sigma = 1.45 \pm 0.05$ mm.;
restoring liver: $M = 15.97 \pm 0.09$ mm.,
$\sigma = 1.94 \pm 0.07$ mm.;

the difference between the means in favor of the restoring liver is 1.85 ± 0.11 mm. For the uninucleate cells:

control: $M = 15.99 \pm 0.09$ mm., $\sigma = 1.84 \pm 0.06$ mm.;
restoring liver: $M = 17.58 \pm 0.12$ mm.,
$\sigma = 2.55 \pm 0.09$ mm. Difference between means
in favor of restoring liver 1.59 ± 0.15 mm.

That the average nucleus of binucleate cells is smaller than the average nucleus of uninucleate cells may be seen from the following:

Difference between nuclei in favor of uninucleate cells:

1.87 ± 0.11 mm. in control series;
1.61 ± 0.15 mm. in the experimental series.

Here again the nuclei of binucleate cells show a high correlation in size ($r = +0.93 \pm 0.01$). The same general conclusions might be drawn from the data on the restoring liver as for those of the control. A count of 500 cells in the hyperplastic liver gave $19.0 \pm 1.1\%$ binucleate cells. This is not significantly different from the $20.8 \pm 1.2\%$ found for the control (difference $1.8 \pm 1.7\%$).

DISCUSSION

It has been known for many years (see reviews by Milne, '09; Fishback, '29; and Higgins and Anderson, '31), that extensive chemical injury, disease or partial removal of the liver is often followed immediately by compensatory hyperplasia. Higgins and Anderson ('31) have shown that in the rat as much as 75% of the liver may be surgically removed

and complete restoration by weight established within 25 to 28 days. In so far as could be determined, animals treated in this way had at the end of complete liver restoration a normal hepatic function.

As previously mentioned the process of restoration is due largely to division of the remaining hepatic cells and by the origin of new hepatic cells from the bile capillaries (Fishback, '29). The method of proliferation has been claimed to be amitotic (Milne, '09; Münzer, '23; Szittyay, '37) or mitotic (Fishback, '29; Higgins and Anderson, '31). Binucleate and large mononucleate cells are thought to arise by amitosis of the nucleus only (Münzer and others) or by the fact that "the nuclei divide promptly by mitosis, but the cytoplasm permits the lapse of varying periods of time before it separates into daughter cells" (the "cytoplasmic lag" of Fishback, '29 and others).

In our work we have never observed evidence of amitosis of the entire cell or of the nucleus only, and therefore cannot support the theory of amitosis as the usual mechanism by which large mononucleate and binucleate hepatic cells arise. Wilson and Deane ('40) have suggested that the so-called amitosis occurring in liver cells "really involves a precise division and distribution of chromatin." Mitotic figures have been observed by several investigators in livers undergoing restoration after poisoning, infection or after experimental extirpation. However, the usual explanation of "cytoplasmic lag" as the method of forming binucleate cells does not seem to be correct. Recent studies have emphasized two integrated aspects of mitosis: karyokinesis (nuclear division) and cytokinesis (cytoplasmic division). It is generally agreed that the mitotic spindle is an important factor in cytoplasmic cleavage; in other words, cells in which cytokinesis depends upon the presence of a karyokinetic spindle do not usually divide under any other condition or at any other time. Therefore the explanation of "cytoplasmic lag" is not in harmony with the facts concerning the mechanism of mitosis. Furthermore, we have demonstrated here that the mechanism in-

volved in forming polyploid mononucleate cells is simply that during mitosis, the cytoplasm fails to divide, thus giving rise to a binucleate cell, with each nucleus possessing the usual diploid complement of chromatin. At the next mitotic division of the binucleate cell, the chromosomes of each nucleus merge on the metaphase plate, resulting at the completion of division in either (1) two large mononucleate (tetraploid) daughter cells, or (2) one large binucleate cell with the octoploid chromatin complement (i.e., each nucleus tetraploid). The latter case arises only when cytokinesis fails to occur. To speak of the failure of the cytoplasm to divide at the time of karyokinesis as "cytoplasmic lag" seems inappropriate because the cytoplasm in our material has never been observed to divide in such cells until the telophase stage of the following division, thus one whole karyokinetic cycle occurs without cytokinesis. It would be interesting to know, however, whether in such material there would be a limit to the number of times the chromosome complement could be doubled; at any rate it seems that a majority of the cells of the normal liver in the one rat studied statistically have become polyploid (i.e., tetraploid).[3] Just what the limiting factors and the individual variations would be in this connection are of course unknown. It might be due, in part, to factors concerned in nuclear-cytoplasmic ratio. Increase in the volume of the nucleus in these cells is accompanied by increase in the volume of the cytoplasm which, however, is extremely difficult to measure accurately.

Jacobj ('25) was able to distinguish in growing mice four classes of liver cells, the volumes of the nuclei (and of the cells themselves) being in the ratio of 1:2:4:8. This, he thinks, is caused by the rhythmic internal doubling of the nucleus and cytosome, but he does not refer to diploid, tetraploid, etc. Binucleate cells are the result of amitosis according to him. Müller ('37) thinks that the changes in size of cells of the liver parallels the development of the cell structure characteristics of that organ.

[3] This result has been confirmed in other rats independently by Norman Sulkin.

No definite evidence as to the cause of the failure of the cytoplasm to divide in these cells during karyokinesis is available from our studies. To speculate on this point we might suggest that it may be due, in part at least, to a large amount of inert materials present in the cell which inhibit the normal cleavage furrows from forming. Storage of glycogen has been reported to take place first in the cells around the central vein; only later is it stored in the peripheral cells. Glycogen is also given up in the reverse order (Maximow and Bloom, '30). If glycogen storage is correlated with the origin of binucleate cells there should be more binucleate cells in the region of the central vein, as has been reported by Münzer ('23). However, Noël ('23) has considered the zone around the central vein as one of repose and the zone in the periphery of the lobule that of permanent function with correlated differences in mitochondria.

We have never observed abnormal karyokinetic figures (i.e., triasters, polyasters, etc.). However, such conditions might be expected in livers that had been affected by disease or poisons. Furthermore, we have never seen more than two nuclei in any one liver cell of the rat but Münzer ('23) reports that as many as seven have been observed for the hepatic cell. MacNider ('36) reports the presence of a syncytial type of atypical epithelial cell in the liver of dogs following injection of uranium nitrate. In this connection treatment of partially hepatectomized animals with poisons such as colchicine should be most interesting.

Münzer ('23) has reported that binucleate cells are relatively uncommon in embryonic and young rabbits up to 12 days old (from 0.2% to 1.1%), while in rabbits from 4 months to about 2 years old the number is greater but relatively constant (15.1% to 29.6%). The same author has presented similar results for the guinea pig, cattle and man (see also Andrew, '41). Increase in binucleate cells in the periphery of the liver lobule has been reported in rabbits following administration of vitamin D (vigantol) by Szittyay ('37) although the specificity of this effect has been denied by Heymann and

Wartman ('41) who found that other substances such as viosterol, inactivated viosterol, dristol, inactivated dristol, propylene glycol, ethyl alcohol and ethyl ether sometimes produced the same effect but with a great variability.

SUMMARY

1. Removal of about two-thirds of the liver of albino rats is followed by rapid mitotic multiplication of cells in that part of the liver remaining. Cytological studies have been made on the excised liver and on the restoring liver 3 days after operation. Amitosis has not been observed.

2. Binucleate cells originate in the liver as a result of the failure of the two presumptive daughter cells to divide during mitosis although the nucleus has divided.

3. Since each of these daughter nuclei is diploid the binucleate cell is tetraploid.

4. The two nuclei of binucleate cells prepare for the next division synchronously, but usually only one spindle is formed at metaphase so that either two tetraploid daughter cells result from a complete mitosis, or cytokinesis may be suppressed again, thus giving rise to a binucleate cell with two tetraploid nuclei.

5. The nuclei of binucleate cells show a high positive correlation in size.

6. There is no significant increase in number of binucleate cells in the restoring liver of the third day as compared with the control liver.

7. Since there is no compensating process whereby the polyploid number of chromosomes is reduced to the normal diploid number, the restoring liver comes to contain a great number of polyploid cells.

8. Cells which are interpreted to have a 16-ploid chromosome number have been found in the restoring liver. No limiting factors controlling the number of suppressed cytokineses which might occur in the lineage of a single cell have been discovered.

9. The fact that the proportion of diploid cells seems to remain relatively constant in the restoring liver may signify that most of the diploid cells undergo complete mitoses, that new diploid cells originate during restoration from buds of proliferating cells sent forth by the bile ducts, or that polyploid cells may be destroyed.

10. The process in the restoring liver probably represents an acceleration of the conditions found in the normal liver. The great variability in size of nuclei and cells in the adult and aging liver may be referred to the suppression of cytokinesis in one or more mitotic cycle.

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EXPLANATION OF PLATES

Photographs of cells of restoring right lateral lobe of the liver of the rat, fixed 3 days after extirpation of median and left lateral lobes. Fixation: Bouin's solution, stain: iron hematoxylin and eosin, except figures 10, 11, and 12 which were stained by the Feulgen method × 1660 (reduced to × 1160 for publication).

PLATE 1

EXPLANATION OF FIGURES

2 Uninucleate cell, diploid.

3 Binucleate cell; each nucleus diploid, cell therefore tetraploid; compare with figure 2.

4 A small (diploid) uninucleate cell on left; large (polyploid) uninucleate cell on right.

5 and 6 Binucleate cells, each nucleus probably tetraploid, cell therefore octoploid.

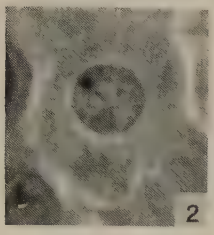
7 Two binucleate cells, both probably tetraploid because of the presence in each of two diploid nuclei.

8 and 9 Uninucleate cells, each nucleus tetraploid, compare with figures 5 and 6.

10 and 11 Binucleate cells showing the synchronous preparation for division of both nuclei in each cell.

12 A large uninucleate cell in preparation for division.

13 Telophase of mitosis of a non-hepatic cell.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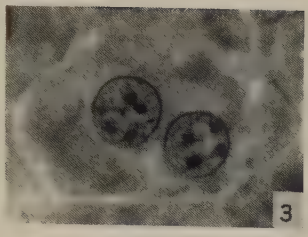
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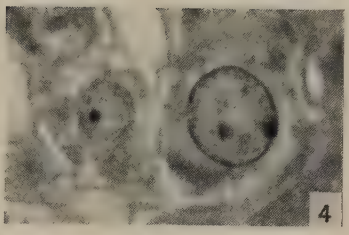
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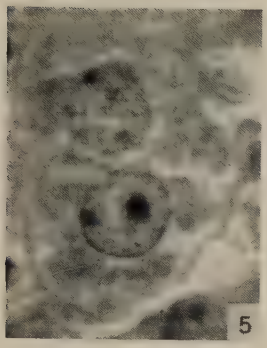
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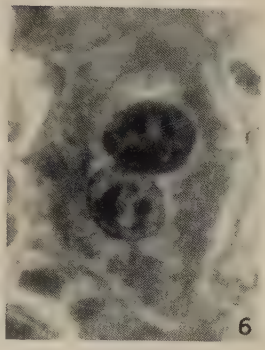
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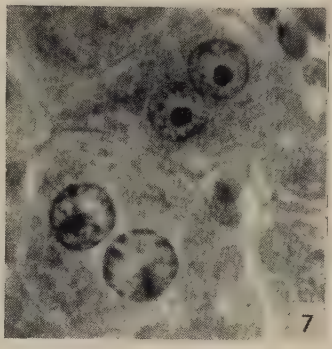
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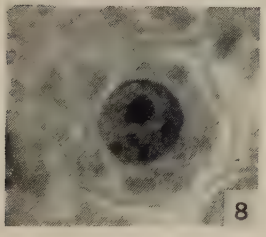
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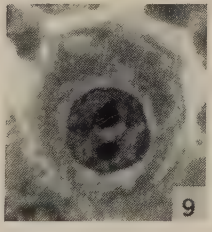
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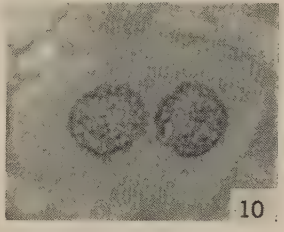
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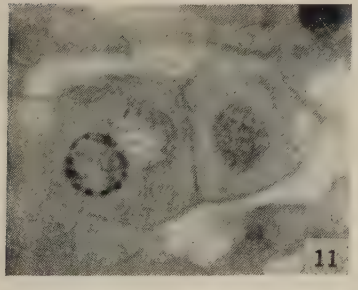
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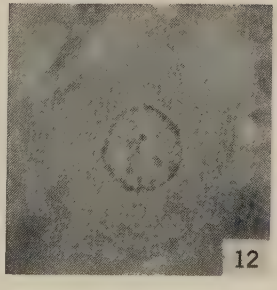
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PLATE 2

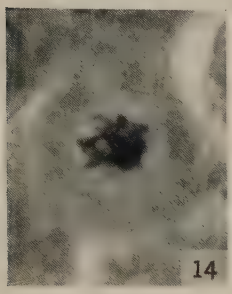
EXPLANATION OF FIGURES

14–18 Stages in division of uninucleate diploid hepatic cells.

19 Late prophase in division of a binucleate hepatic cell. Chromosomes forming one equatorial plate.

20 Telophase of cell which is apparently not to undergo cytokinesis.

21–24 Stages in division of polyploid hepatic cells.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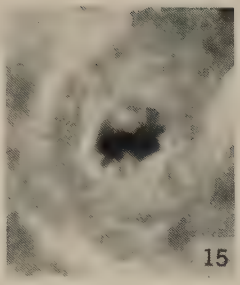
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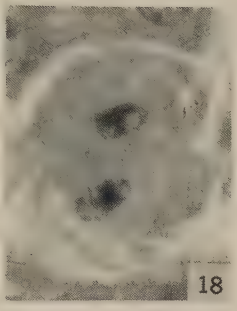
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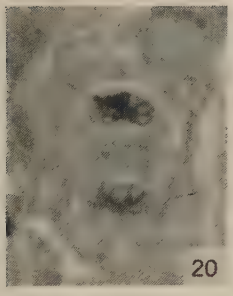
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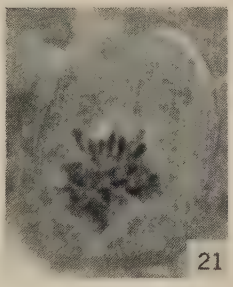
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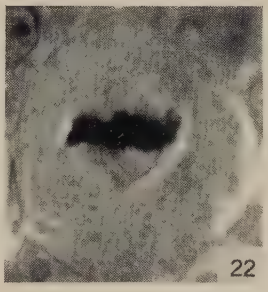
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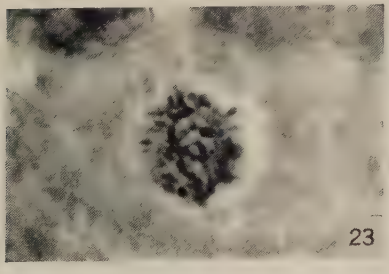
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SURVIVAL OF SPERMATOZOA IN THE FEMALE REPRODUCTIVE TRACT OF THE BAT

WILLIAM A. WIMSATT

Laboratory of Histology and Embryology, Department of Zoology, Cornell University, Ithaca, New York

ONE PLATE (FIVE FIGURES)

Previous studies of the reproductive phenomena in certain hibernating bats (Vesperugo pipistrellus, Vespertilio murinus, Plecotus auritus, Vesperugo noctula, Rhinolophus ferrum-equinem) have established the following facts: (1) In the male vespertilionid and rhinolophid bats spermatogenesis takes places in the summer and ceases in the early fall, at which time the seminiferous tubules undergo involution and remain in a quiescent state until the following spring. During the hibernation period the cauda epididymis is distended with numerous spermatozoa, and the accessory glands are in a secretory state (Courrier, '27; Nakano, '28; Miller, '39). (2) Copulation takes place in the autumn as the bats enter into hibernation and it may occur periodically any time thereafter through the winter and early spring whenever the animals are aroused (Fries, 1879; Eimer, 1879; Guthrie, '33, and the writer who has observed abundant active spermatozoa in vaginal smears made from Myotis l. lucifugus and Eptesicus f. fuscus taken in October and November). (3) The spermatozoa of the fall inseminations are stored during hibernation either in the uterus in the family Vespertilionidae (Robin, 1881; Courrier, '27; Nakano, '28), or in a ventral outpocketing of the vagina in the Rhinolophidae (Fries, 1879; Courrier, '27). (4) Ovulation occurs concomitant with resumed activity in the spring (Guthrie, '33).

The question long at issue is: Are these vernal ova fertilized by the spermatozoa of the fall matings, or are spermatozoa of winter and early spring matings necessary for their activation?[1]

Although fragmentary, the observations of workers previous to 1933, almost without exception, support the view that the spermatozoa of the fall copulations activate the vernal ova. The reports of Nakano ('28) and Redenz ('29) show that in the uterus of the hibernating bat the spermatozoa are embedded in a gelatinous matrix which inhibits their movement. This has been interpreted as a mechanism which functions to conserve the vitality of the sperm over the winter sleep. The literature need not be reviewed in detail here since is was summarized by Hartman in 1933.

Since 1933, however, investigators have made observations which seem to cast doubt upon the ability of spermatozoa which have survived from the fall matings to activate eggs released from the ovary in the spring. Guthrie ('33) found that sperm were able to survive in the uterus of Myotis for at least 1 month, but discovered that periodic activity was correlated with a disappearance from the female genital tract of spermatozoa introduced in the autumn. She decided that late winter and early spring copulations are necessary to fecundate the vernal ova. The work of Guthrie and Jeffers ('38) lends further support to this idea. They studied the presence of developmental stages in the tubes and uterus of two species of Myotis (M. l. lucifugus and M. grisescens) following injections of hypophyseal extract in different months of the hibernating season, and concluded that the spermatozoa of the fall inseminations probably are not concerned in the activation of the vernal ova, but that the sperm of the late winter and early spring matings are responsible.

[1] Several early European workers (Hartman, '33) have reported observing copulation during the winter and spring among a number of species of continental hibernating bats. In America, Hartman and Cuyler ('27) and Guthrie ('33) have reported observing winter copulations in Myotis, and Mr. H. Trapido and the writer have made similar observations for Myotis subulatus leibii and Myotis l. lucifugus in December, January, and February.

Gates ('36), on the other hand, furnishes the first evidence that sperm which have remained a long time in the female reproductive tract are able to initiate development. He autopsied, on February 10th and 11th, four female bats of two species, Myotis sodalis and M. l. lucifugus, which had been isolated from males since December 4th (68 days). Two specimens of M. sodalis each contained a single embryo slightly over 1 mm. in length. Of the two remaining M. l. lucifugus, one possessed a single embryo, the other, two embryos nearly a millimeter in length. Fertilization was therefore accomplished by spermatozoa introduced into the vagina and uterus before December 4th, the date of isolation.

Folk ('40) found in the right uterine horn of a female big brown bat, Eptesicus fuscus fuscus, isolated in January and autopsied in April, two embryos, the younger of which was in the late primitive streak stage. His experiment shows that spermatozoa which have remained in the uterus approximately 90 days are fully capable of performing their normal function.

Because of the conflicting evidence, and the paucity of conclusive results, further studies appear desirable. The problem of sperm survival in the bat can be finally solved only by isolating females in the autumn to obviate possible winter copulations, and searching, in the spring, for spermatozoa or embryos in their genital tracts. The writer has undertaken these studies, and he is not only prepared to show that spermatozoa may survive within the female genital tract throughout the period of hibernation, but he has secured further evidence that spermatozoa of the fall inseminations are able to initiate development of ova released from the ovaries in the spring.

The writer wishes to express his appreciation to Prof. Howard B. Adelmann who examined the material and read the manuscript, and to Mrs. Ruth Wimsatt who likewise read the manuscript, and both of whom made suggestions for its improvement.

OBSERVATIONS

Throughout the fall and winter of 1940 and 1941 semimonthly trips were made to Aitkin cave in Mifflin County,

Pennsylvania, for the purpose of collecting histological material and continuing certain ecological studies on the hibernating bats. Several groups of females of Myotis l. lucifugus and Eptesicus f. fuscus were brought back to the histological laboratories at Cornell University, isolated from any males, and placed in an electric refrigerator where a temperature of 38° to 42° F. was constantly maintained. The bats were confined in 12″ × 10″ × 18″ wire cages which were placed in water pans, the wire bottoms of the cages barely touching the surface of the liquid. The humidity was further augmented by damp towelling placed over a portion of each cage.

The animals remained undisturbed throughout the winter and little mortality occurred before spring. Most investigators

TABLE 1

Length of life of spermatozoa in the female genital tract.

NO.	SPECIES	HIBERNATION PERIOD	AUTOPSY	SPERM IN UTERUS
1	M. l. lucifugus	11/17/40 to 4/18/41	3/10/41	Abundant and active
2	M. l. lucifugus	11/17/40 to 4/18/41	3/25/41	Abundant and active
3	M. l. lucifugus	11/17/40 to 4/18/41	4/21/41	Abundant and active
4	M. l. lucifugus	12/ 1/40 to 4/18/41	4/21/41	Abundant and active
5	M. l. lucifugus	12/14/40 to 4/18/41	4/18/41	Abundant and active

have held that the sperm of the autumnal matings are able to survive within the female genital tract over the winter, but until the experiment of Folk no one had actually succeeded in confirming this belief. Consequently at intervals through the winter the writer sacrificed a specimen, made smears in physiological saline solution of the uterine scrapings, and examined these under the microscope for the presence or absence of spermatozoa. Almost invariably such preparations were found to contain numerous active sperm.

Table 1 is a summary of the data on five specimens of Myotis l. lucifugus, and represents the condition typical of most bats of this species which were examined. The animals were isolated from males on the first date in the column entitled "Hibernation period."

Since there was no opportunity for copulation in these segregated females, this table shows that spermatozoa are able to survive in the uterus for over 5 months (156 days in specimen no. 3). That such a condition exists also in Eptesicus f. fuscus, is suggested by the experiment of Folk previously described, and the observations immediately to follow.

While the longevity of spermatozoa in the female reproductive tract of the hibernating bat can no longer be doubted, the ability of these sperm to initiate normal development has been questioned (Guthrie and Jeffers, '38). No results are yet available for Myotis l. lucifugus, but some interesting observations are at hand for the big brown bat.

Seven specimens of Eptesicus f. fuscus survived the period of artificial hibernation and were killed at intervals in the spring. The reproductive tracts of all were fixed in Bouin's solution, sectioned at 10 μ, stained in Harris's hematoxylin and eosin, and examined under the microscope for pregnancy or evidence that ovulation had taken place.

Ovulation had occurred in only two of these females, whereas the ovaries of four of the remaining five bats showed enlarging follicles of various sizes, ranging from 100 μ in diameter to follicles approaching rupture (400 μ). One specimen was immature.

Both of the bats wherein ovulation had occurred were collected on December 14, 1940. They were immediately isolated from males and subjected to artificial hibernation in the laboratory. On April 18, 1941 the animals were removed to a warm room having an average temperature of 75° F., where they were kept in small semi-dark cages, supplied with an adequate amount of water, and fed daily on small pieces of beef liver.

The first to be autopsied (specimen J) was killed on May 7, 1941 while in a somewhat weakened condition. Upon gross examination the uterus and the oviducts were seen to be slightly enlarged, and the ovaries gave evidence of recent ovulation. Microscopic examination of the uterus revealed four embryos in early stages of cleavage, with three to five

blastomeres each (fig. 2). Two were present in each uterine horn. Likewise two corpora lutea were present in each ovary, but the luteinizing granulosa cells had not completely filled the evacuated antra. The uterine mucosa was markedly different from the usual non-gravid condition; the epithelium was higher, and the glands had proliferated and increased in number.

The second female (specimen N) was autopsied on May 19, 1941. Here again the uterus was enlarged (fig. 1) and the ovaries showed signs of recent ovulation. Under the microscope three segmenting ova were found in the uterus; two in the right horn, and one in the corpus. The ova consisted of approximately thirty to thirty-five cells each (fig. 3). Microscopic examination of the ovaries disclosed two corpora lutea in the right ovary and one in the left (fig. 4). These were in the same stage of development as those of specimen J, and the condition of the uterine mucosa was likewise similar. In both specimens the uterine glands were in the secretory state.

Even in the early stage represented in specimen J, the adjacent lining of the uterus appears to have reacted to the presence of the ova. Each ovum lay in a locally enlarged portion of the uterine cavity, characterized by a marked thinning of the epithelium in the immediate vicinity of the egg (fig. 2). The significance of this is not yet well understood and is being investigated in connection with studies on development now in progress.

Undoubtedly the sperm responsible for the activation of these ova remained within the female reproductive tract throughout the period of artificial hibernation; in specimens J and N approximately 140 and 150 days respectively.

This fact not only shows indirectly that spermatozoa may remain alive for long periods of time within the uterus in Eptesicus, but it also indicates that in the two specimens discussed, the spermatozoa of the autumnal matings, which had remained in the female reproductive tract over the hibernation period, were capable of initiating development of the eggs released from the ovary in the late spring.

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PLATE 1

EXPLANATION OF FIGURES

1 Pregnant uterus; ventral aspect of specimen N. × 18.

2 Longitudinal section of specimen J showing a cleaving ovum in the 3-cell stage. Also visible is the thinning of the epithelium of the uterus due to the presence of the ovum. × 370.

3 Transection of right uterine horn of specimen N showing a section of a cleaving ovum of approximately thirty cells. × 310.

4 Two corpora lutea in the right ovary of specimen N. The luteinizing granulosa cells have not filled the antra. × 109.

5 Corpus luteum cells from figure 4 shown under greater magnification. × 198.

Photographs by Arthur L. Smith.

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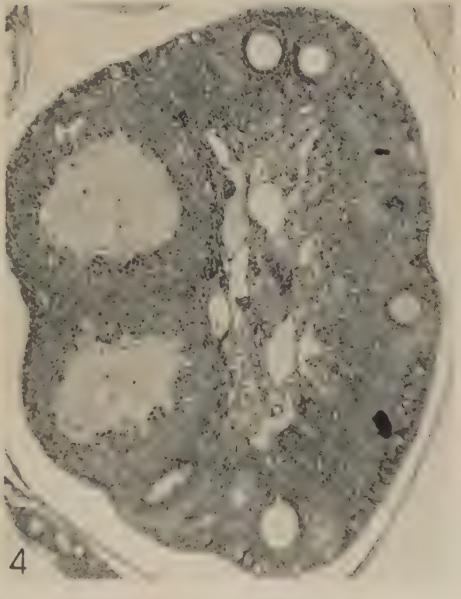
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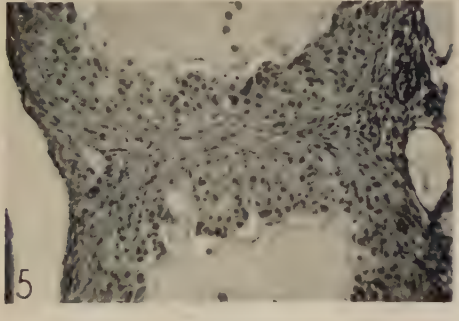
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STAGES IN THE NORMAL DEVELOPMENT OF RANA PIPIENS

II. IDENTIFICATION OF THE STAGES FROM SECTIONED MATERIAL

WALDO SHUMWAY[1]
Department of Zoology, University of Illinois, Urbana

TWO PLATES (TWENTY-FIVE FIGURES)

This is the second of a series describing normal stages in the development of Rana pipiens. Its purpose is to afford a method of identifying from sectioned material the stages described in the first communication (Shumway, '40). Typical sections through carefully staged embryos are described briefly and illustrated by photomicrographs.

All embryos were fixed in Smith's ('15) frog fluid for 24 hours and washed in 5% formalin. The egg jelly was removed in a 10% solution of Clorox. After dehydration in successive concentrations of alcohol, the embryos were cleared in benzene and embedded in tissuemat. Serial sections were cut in each of the customary planes at a thickness of 20 microns.

Most sections were stained with Feulgen's nucleal reaction. The most satisfactory results were obtained by hydrolyzing 45 minutes and staining for 1 hour. After a thorough washing in sulfurous acid water and distilled water, they were counterstained with picro-indigocarmine, dehydrated, cleared and mounted in clarite. Figures 8, 9, 10, 11, 24, 25 are from slides stained in Delafield's hematoxylin and eosin.

Photographs were made (except 1 A) at an initial magnification of 14 ×, using a Zeiss planachromat 3 × lens and a Phoku camera. Eastman Panatomic X cut film was employed

[1] With the technical assistance of George Svihla.

and developed in Eastman D-76. The positives, made at an additional magnification of 2.4 ×, have been reduced in reproduction to the magnification indicated for the plates. Figure 1-A was made at an initial magnification of 250 ×.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

(All figures except 1 A × 16½)

1 Stage 1. Unfertilized egg. Vertical section. This shows the fovea germinativa and the region of the mitotic spindle preparatory to the second meiotic division.

1 A Stage 1. Same section as 1. Detail of fovea germinativa showing chromosomes and spindle fibers, also distribution of yolk and pigment granules in vicinity of animal pole. × 450.

2 Stage 2. Fertilized egg. Transverse section showing (white spot in upper left quadrant) clear area surrounding the pronuclei which are now in apposition. The dark area above and to the right of the clear area is pigment marking the trail of the sperm pronucleus.

3 Stage 3. Two-cell stage. Transverse section. This shows the nucleus in the right hand blastomere, pigment surrounding the remnant of the mitotic spindle and the rounded edge of the cleavage furrow.

4 Stage 4. Four-cell stage. Transverse section showing the first cleavage furrow, above, and the space created by the second cleavage furrow.

4 A Stage 4. Frontal section at level of nuclei. The first cleavage furrow is now flattened while the second is widely open. A slight indication of the polar furrow is to be seen. The extent of this furrow varies greatly in individual specimens. The area of the gray crescent, marked by lighter surface pigmentation is to the right. The nuclei are in the metaphase in this specimen but often are found in the anaphase.

5 Stage 5. Eight-cell stage. Sagittal section. The cavity is that of the first cleavage furrow. Second and third cleavage furrows are indicated by grooves. The gray crescent is to the left.

6 Stage 6. Sixteen-cell stage. Parasagittal section. Indications of nuclei in each of the blastomeres of the animal hemispheres suggest bilaterally sym-

metrical cleavage. The extent of the segmentation cavity would be much greater in a sagittal section. The gray crescent is to the right.

7 Stage 7. Thirty-two-cell stage. Sagittal section. Gray crescent to the right. Although the cleavage furrows are exaggerated in preparation (I have no thoroughly satisfactory sections at this stage), the smaller size of the blastomeres in the gray crescent area is apparent.

8 Stage 8. Mid-cleavage stage. Sagittal section. Gray crescent to the right. The excentricity of the blastocoel is evident. It will be noted that the side wall is thinner in the area of the gray crescent. The pigment around the nuclei is very striking.

9 Stage 9. Late cleavage stage. Sagittal section. Gray crescent area to the right. The roof of the blastocoel is much thinner than in the preceding stage. The thinner wall of the blastocoel is on the gray crescent side and the cells in the gray crescent area (marginal zone) are smaller.

10 Stage 10. Dorsal lip stage. Sagittal section, gray crescent area to the right. The dorsal lip is indicated by a slight groove and heavy pigmentation 25° below the equator. This is definitely higher than the position of the dorsal lip shown for the corresponding stage of Discoglossus pictus (Dalcq, '38). The first appearance of the dorsal lip is in the white area and not at the junction of the pigmented and white areas as often stated. The roof of the blastocoel is still thin, and the gray crescent side thinner than the other side walls. There is no evidence of ciliation in R. pipiens at this stage.

11 Stage 11. Lateral lip stage. Sagittal section, dorsal lip to right. The dorsal lip has advanced to approximately 30° below the equator but only a small amount of invagination has taken place. There is a considerable amount of pigment in the region of invagination. The pigment extends almost to the point where the ventral lip will make its appearance. The delamination of the ectoderm into an epidermal and a nervous layer seems to be exaggerated in preparation. The roof and slides of the blastocoel are now of the same thickness. The preparation seems to show the phenomenon described by Brachet ('03) as "gastrular cleavage."

12 Stage 12. Ventral lip stage. Sagittal section, dorsal lip to right. The ventral lip has just made its appearance, indicated by a slight groove and pigmentation approximately 60° below the equator. The dorsal lip has also advanced to about the same position on the opposite side. The slit-like gastrocoel (archenteron) extends from 15° above the equator to 55° below the equator and the advancing anterior wall of this cavity has limited the blastocoel to the anterior ventral portion of the embryo.

13 Stage 13. Neural plate stage (neurula). Sagittal section, blastopore to right. A small yolk plug is apparent in surface views but all sections indicate that this has been detached from the inner mass of yolk-laden cells. The embryo has rotated 90° to bring the yolk plug to a posterior position. Meantime the gastrocoel has enlarged very greatly but the blastocoel is still present, A very thin layer of cells separates the two cavities and this is easily destroyed in preparation. The blastocoel can be traced as a separate cavity until stage 15. The roof of the gastrocoel is composed of three layers, epidermal ectoderm, nervous ectoderm and chorda-mesoderm, respectively.[2]

[2] An interpretation of figures 10, 11, 12, 13, and 13 A, 14 A and 15 A has been presented in the fourth edition of my Vertebrate Embryology.

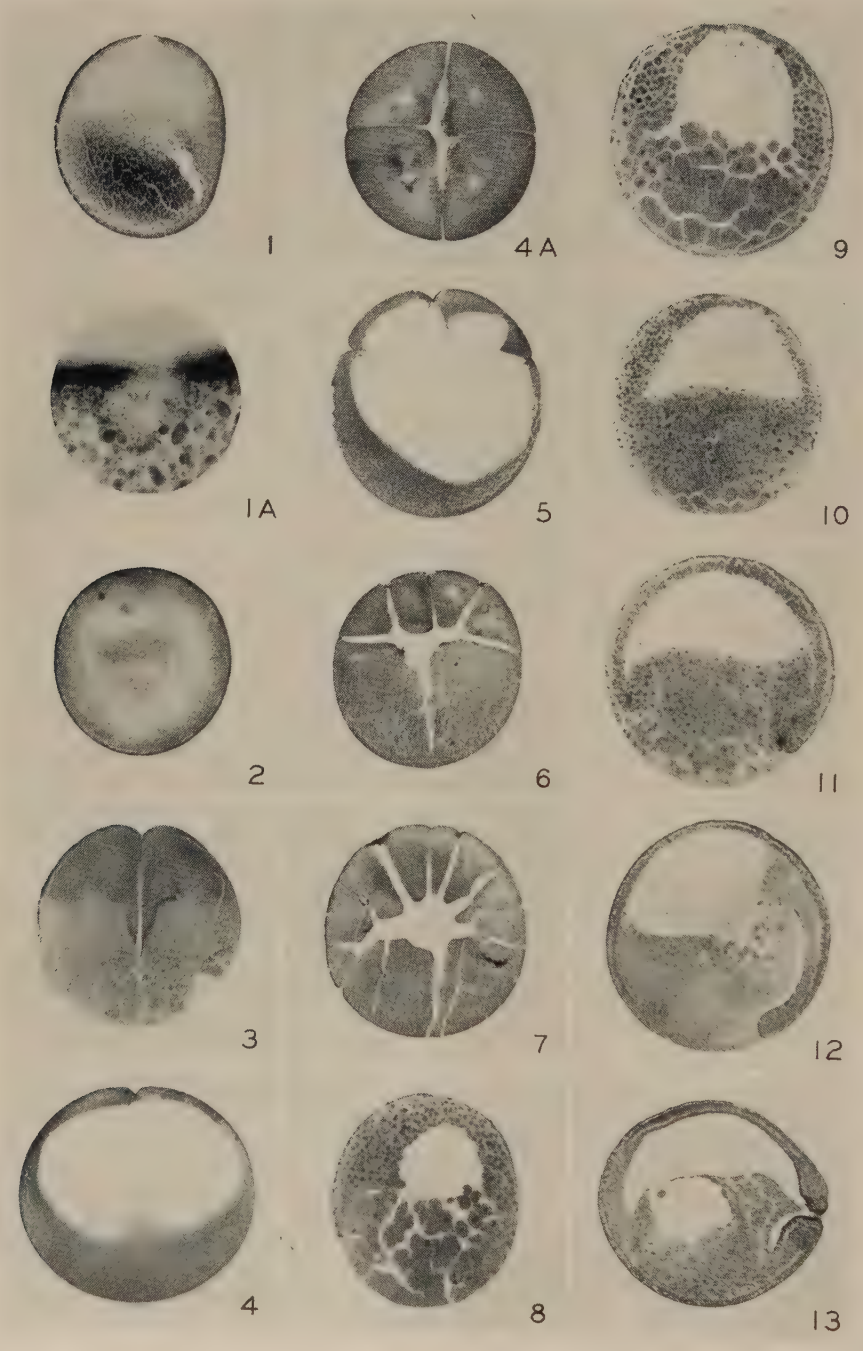
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PLATE 2

EXPLANATION OF FIGURES

(All figures magnified × 22½)

13 A Stage 13. Neural plate stage. Median transverse section. The depth of the gastrocoel is roughly one-third the diameter of the embryo. The thickening of the nervous layer of the ectoderm is greatest at the margin of the neural plate. At this stage the endoderm has begun to advance upward over the chordamesoderm which at this time constitutes the roof and sides of the gastrocoel. A slight thickening and flattening of the roof indicates the position of the notochord.

14 Stage 14. Neural fold stage. Transverse section through the optic field. The nervous layer of the ectoderm is greatly thickened and a trough-like depression marks the beginning of the neural groove. The site of the future optic vesicles is marked by heavy pigmentation and elongation of the cells. I have been unable to find any transverse sections resembling Eycleshymer's (1893) well known figure of Rana palustris but have seen a similar section cut in a plane intermediate between transverse and frontal at a stage midway between my stages 13 and 14.

14 A Stage 14. Median transverse section. During preparation the neural folds become flatter than they appear in surface views of living specimens and the neural groove also is shallower. The elevation of the neural folds appears due to the nervous layer of ectoderm which has shifted its position towards the midline. The wings of endoderm have advanced upward and have united with the lower portion of the notochord. This meantime has separated from the axial mesoderm on the dorsal side but is still connected with it ventrally. The region where notochord, mesoderm and endoderm are united will later separate to form the hypochord. There is still a mesodermal lacuna on the ventral side. The small cavity is the remnant of the blastocoel.

15 Stage 15. Stage of rotation. Transverse section through developing optic vesicles. In preparation the neural folds have separated slightly whereas in life they are in apposition. The thickness of the nervous layer is well marked as is the pigmentation and columnar form of the cells which will form the optic vesicles. The cavity is that of the fore-gut.

15 A Stage 15. Median transverse section. The divergence of the neural folds is an exaggeration produced in preparation. The axial mesoderm is completely separated from the notochord but the latter is still united with the primordium of the hypochord. The restriction of the mid-gut due to the thickening of the endodermal side walls is apparent. The blastocoel has disappeared. Although not shown in the photograph ciliated cells have now made their appearance in the ectoderm and the embryo rotates slowly in its egg jelly. In frontal sections 3 and 4 somites may be counted.

16 Stage 16. Neural tube stage. Transverse section through optic vesicles. The characteristic at this stage is the uniform thickness of the wall of the optic vesicle with that of the fore-brain. The heavy pigmentation and columnar form of the cells composing the primordium of the oral suckers are evident. The section appears to be slightly posterior to that shown by Pollister and Moore ('37) for the corresponding stage of Rana sylvatica and the cavity of the vesicle is larger. There are 6 to 7 somites in frontal sections.

17 Stage 17. Tailbud stage. Section through optic vesicle. The wall of the vesicle is now characterized by the thinness of the dorsal surface which is to form the pigmented layer (tapetum nigrum) of the retina. The lens placode is not

yet apparent. The cavity is that of the fore-gut. In more posterior sections the suckers appear cup-shaped in cross section. There are 12 or 13 somites. The thinness of the future pigmented layer as compared to the future sensory layer of the retina is more marked than in Rana sylvatica.

18 Stage 18. Stage of muscular response. Section through optic cup. The head is now laterally compressed. The invagination of the sensory layer of the optic cup has proceeded until it is in contact with the pigmented layer, now very thin and showing pigment. The lens primordium is now visible as a placode. The oral suckers are closer to the median line and in more posterior sections appear as well developed cups. There are now 18 or 19 somites.

19 Stage 19. Stage of heart beat. Section through optic cup. The lens placode has formed a shallow pit which is characteristic of this stage. The oral sucker reaches its maximum development in this stage. There are 23 to 25 somites. Judging by the section this stage is a little more advanced than stage 19 of Rana sylvatica, in which the lens placode shows no invagination and the number of somites is 20 (Pollister and Moore, '37).

20 Stage 20. Stage of circulation in first gill bud. Section through optic cup. In this stage the embryo usually hatches at 18°C. The optic cup is more rounded and the lens is now a vesicle with a small central cavity and still connected by a slight stalk to the overlying epidermis.

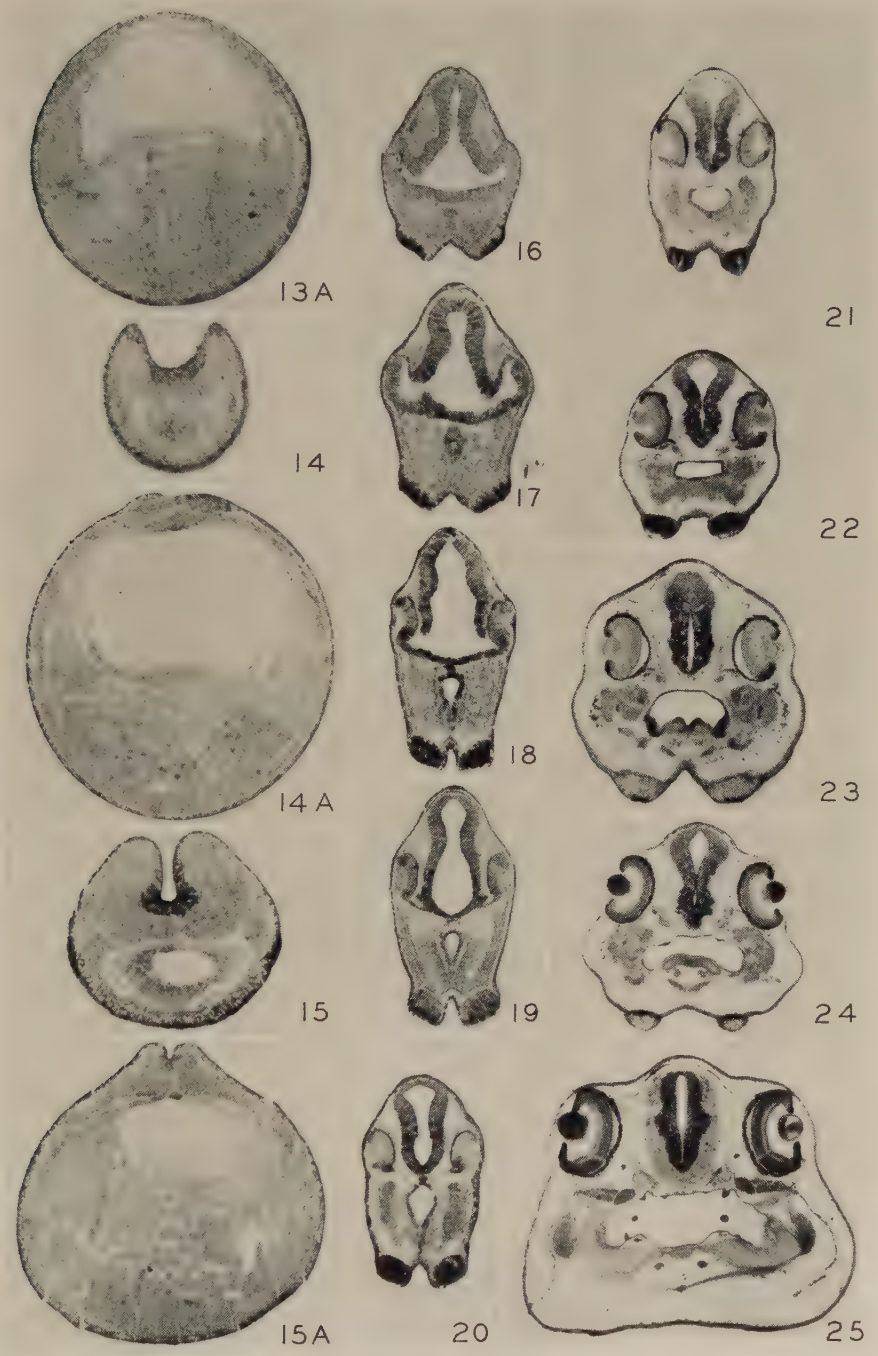
21 Stage 21. Stage of transparent cornea. Section through eye. In this stage the mouth is open and spontaneous swimming begins. The proportions of the head have undergone a marked change in which the lateral compression is not so evident. In the lens of the eye the elongation of the cells making up the inner wall affords a diagnostic character. The regression of the oral sucker is noticeable.

22 Stage 22. Stage of tail fin circulation. Section through eye. The eye and its lens are now larger in proportion to the size of the head which is almost round at this time. The inner wall of the lens almost fills the cavity of the lens vesicle. In the retina a cellular layer and fibrous layer can be distinguished.

23 Stage 23. Opercular fold stage. Section through eye. The head is much larger. In the retina of the eye two cellular layers, nuclear and ganglionic respectively can be distinguished. The separation of the pigmented layer of the retina is an accident of preparation. It may be noted that the inner layer of the lens vesicle has not yet fused with the outer wall.

24 Stage 24. In this stage the operculum is complete on the right side. The individual selected for illustration appears undersized due to shrinkage in preparation. The eyes now protrude slightly from the sides of the head, not so much as would be indicated in this figure due to shrinkage. The lens cavity has been obliterated and the nuclear layer of the retina is beginning to separate into inner and outer layers. The oral suckers are now represented only by two small pigmented protuberances on the ventral side of the head.

25 Stage 25. Operculum complete. The head of the embryo is now much larger as are the protuberant eyes. Rods and cones appear in the retina. The suckers are reduced to small pigmented patches.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BILATERAL HYPERTHELIA IN A GUINEA PIG

HAROLD SPEERT

Carnegie Institution of Washington, Department of Embryology, and Department of Obstetrics, Johns Hopkins Hospital, Baltimore, Maryland

ONE FIGURE

Number of mammary glands is a species character of mammals and is correlated, in a general way, with the average litter size of each species. When more than this average number of glands or nipples is observed, the additional ones are referred to as supernumerary structures. Such supernumerary or accessory mammae are encountered with different degrees of frequency among the various species. The guinea pig, which usually bears two or three young to the litter, normally possesses but a single pair of mammary glands, which are located in the inguinal region. Supernumerary nipples apparently occur with great rarity, for insofar as the writer is aware there are only two recorded instances of extra nipples in this animal (Turner and Gomez, '33). It is the purpose of the present report to place on record what is believed to be a unique case of bilateral supernumerary nipples in the guinea pig.

A mature female guinea pig was acquired from an animal dealer in Baltimore during the summer of 1939. She was mated in the laboratory during the fall of the same year. Toward the end of the resulting pregnancy an extra nipple was noted in the left pectoral region. When this area was shaved the supernumerary nipple was found to be surrounded by a small, deeply pigmented areola. In addition, a similar although somewhat smaller nipple and areola were found in the right pectoral region, slightly below the level of the left super-

numerary. The pregnancy was allowed to continue uneventfully to term, when two normal young were delivered (December 11, 1939). Lactation ensued in a normal fashion from the two normal inguinal glands. No milk could be expressed from the supernumerary nipples, however, and there was no palpable mammary parenchyma associated with them. The accompanying photograph was taken on the fourth postpartum day. At this time the left accessory nipple was excised, and the absence of glandular tissue was confirmed by dissection under the binocular microscope. Histologic examination of the longitudinally sectioned nipple revealed the characteristic nipple structure, including a central duct.

The remaining accessory nipple was painted daily (except Sunday) with an alcoholic solution of estrone (0.5 mg. per cubic centimeter) from December 20, 1939 to May 15, 1940, a total of 147 days. Little or no growth was observed during this period. Similar treatment had previously been found effective in producing localized unilateral nipple growth in castrated male guinea pigs and prepubertal male monkeys (Lamar and Speert, unpublished), with development of the underlying mammary gland (Speert, '40). In the rhesus monkey it was also found capable of causing growth of supernumerary nipples (Speert, '42). The failure of additional development of the guinea pig's supernumerary nipple may be explained on the basis of its already having experienced its maximal growth possibilities in response to the estrogenic stimulation of the recent pregnancy. In pregnant monkeys a similar absence of nipple response to locally applied estrogens was observed.

The hereditary nature of supernumerary nipples and mammary glands is well established. The familial occurrence of these anomalies has been recognized in sheep (Castle, '24), swine (Wentworth, '16), chimpanzee (Elder, '36), and monkey (Speert, '42), as well as in man. Despite careful examination of the two infants born to the guinea pig described in this paper, however, no evidence of supernumerary mammary structures could be found.

In an exhaustive tabulation of the literature concerning the number and morphology of mammary glands in the various species of mammals, Schickele in 1899 remarked that he had never seen more than two nipples in an adult guinea pig. In the composite experience of Cuvier, Giebel, and Gegenbauer likewise, never had more than two nipples been encountered.

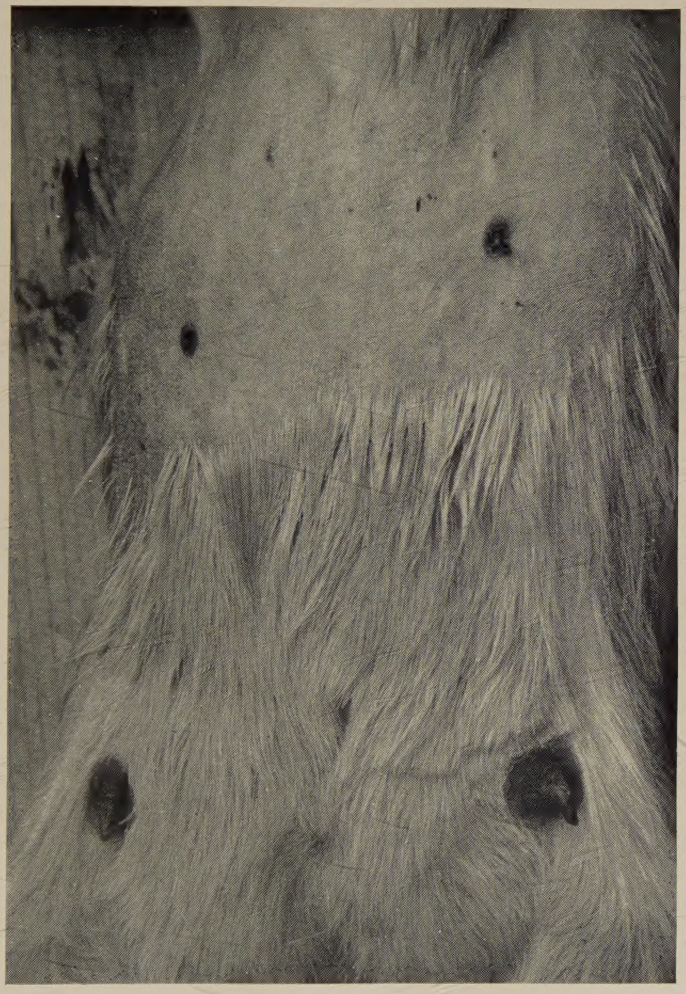

Fig. 1 Guinea pig with two supernumerary nipples, showing their relation to the normal inguinal mammae.

Turner and Gomez ('33) reported, for the first time, two guinea pigs, a male and a female, each of which possessed an additional nipple. The present case is therefore the third recorded instance of hyperthelia in the guinea pig, and the first in which supernumerary nipples were present bilaterally. In the human, by contrast, supernumerary mammary structures occur with an incidence of at least 1%.

Schickele's early study revealed the presence of from two to ten supernumerary mammary rudiments in guinea pig embryos. These were arranged along the milk line and probably corresponded to the "primitive Zitzen" of Schultze (1892). They invariably disappeared during later stages of development and left no suggestion of their former presence at birth. The presence of supernumerary mammary structures in postnatal life may therefore be interpreted as an abnormal persistence of these embryonic rudiments.

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